THYROID HORMONE AVAILABILITY DURING PREGNANCY AND EARLY LIFE: DETERMINANTS, INTERPRETATION AND CONSEQUENCES

TRANSLATING THYROID PHYSIOLOGY INTO CLINICAL EPIDEMIOLOGY STUDIES.

Tim I. M. Korevaar

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Prof.dr. E.A.P. Steegers

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Prof.dr. H. Tiemeier
Dr. K. Boelaert

Copromotor: Dr. Marco Medici

Paranimfen: Drs. P. F. Vollebregt
Drs. L. Chaker

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GENERAL INTRODUCTION

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Tim I.M. Korevaar and Robin P. Peeters
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THYROID FUNCTION IN PREGNANCY

Thyroid dysfunction during pregnancy is common with a prevalence of 2-4%.[1,2] Maternal thyroid dysfunction is associated with an increased risk of various adverse maternal and child outcomes, including miscarriage, intra-uterine growth retardation, hypertensive disorders, preterm delivery, and a decreased child IQ.[2-4] During pregnancy, profound changes in thyroid physiology occur in order to provide sufficient thyroid hormone (TH) to both the mother and fetus. This is particularly important during early pregnancy as the fetal thyroid only starts to produce considerable amounts of TH from approximately 20 weeks of gestation, until which the fetus heavily depends on the maternal supply of TH. This supply of TH to the fetus, as well as increased concentrations of TH binding proteins (thyroxine-binding globulin; TBG), and degradation of TH by placental type 3 iodothyronine deiodinase (D3) necessitate an increased production of maternal TH.[1,2] This requires an intact thyroid gland and adequate availability of dietary iodine, and is in part mediated by the pregnancy hormone human chorionic gonadotrophin (hCG), which is a weak agonist of the thyroid-stimulating hormone (TSH) receptor.[5] As a consequence, serum free thyroxine (FT4) levels increase and TSH levels decrease from approximately the 8th week throughout the first half of pregnancy, resulting in different reference ranges for TSH and FT4 compared to the non-pregnant state.

Given these pregnancy related changes in thyroid physiology and the complications associated with thyroid dysfunction, it is of importance to determine reference ranges for a normal thyroid function during pregnancy. This is crucial to identify women that would potentially benefit from treatment. For this reason, the guidelines of the Endocrine Society, American Thyroid Association and European Thyroid Association recommend to calculate trimester-specific reference ranges per center.[6-8] If these calculated ranges are not available in the laboratory, TSH reference ranges of 0.1–2.5 mU/L for the first trimester and of 0.2–3.0 mU/L for the second trimester are recommended.[6-8] These reference range estimations were based on the published reference ranges of 6 pregnancy cohorts.[9-14] Even though many additional pregnancy cohorts have published their center-specific reference ranges since the publication of these guidelines, showing substantial differences in cut-offs for TSH, most institutions still rely on these fixed reference ranges. This is particularly relevant since even subclinical thyroid dysfunction, mostly defined according to population-based cut-offs, is associated with an increased risk of adverse maternal and child outcomes.

THE CONTINUOUS SPECTRUM OF THYROID HORMONE ACTION DURING EARLY LIFE

Well recognized thyroid disease entities such as Hashimoto's thyroiditis, cretinism and congenital hypothyroidism have taught us many things about thyroid hormone action during early life. Although thyroid disease is generally considered a dichotomous outcome, it has become clear that also subclinical forms of thyroid dysfunction, and variation within the normal range, may resemble the clinical phenotype of overt disease, albeit in a milder form. This is illustrated by studies showing that pregnant women with a suboptimal thyroid function, including subclinical forms of disease but also low, or high-normal thyroid function, have offspring with a lower IQ and a higher risk of neurobehavioral disease.[15-17]

The importance of such findings is highlighted by the fact that the incidence of subclinical thyroid disease is at least 10-fold higher than overt thyroid disease. Notably, neurobehavioral outcomes have always been of special interest because the brain is a target organ for thyroid hormone and already small differences in neurobehavioral outcomes can have major public health consequences.[18] However,

investigating the consequences of subclinical forms of thyroid disease is difficult because milder phenotypes are not always that obvious. Consequently, the potential adverse effects need to be actively investigated and large numbers are needed to show relatively small effects.

A study by Samantha Lain and colleagues is the first large epidemiological study that suggests a link between the biochemical phenotype of a subclinical form of congenital hypothyroidism and suboptimal brain development. The extensive data and dose response relationships demonstrated in their work add to our knowledge on the risks associated with inadequate thyroid hormone availability during early life. Moreover, their results may indicate that we can optimize congenital hypothyroidism screening by reconsidering thresholds for follow-up.

The outcome of neurobehavioral tests are influenced by many factors which makes these tests notoriously prone to measurement error. For educational outcomes as a marker of neurobehavioral development, measurement error is likely to be even higher and therefore studies investigating such outcomes require a large study population. Recent studies that were relatively small have failed to show any consistent association of fetal or neonatal thyroid hormone availability with similar school outcomes.[19,20] By linking individual records from multiple data sources in Australia, Lain and colleagues were able to study over 500.000 children. The authors investigated the association of neonatal TSH concentrations, obtained during heel-prick screening, with educational results for numeracy or reading at age 7-15 years (N=354.137), as well as being vulnerable in developmental domains or having special needs at age 4-6 years (N=149.569). The results of the study show a positive dose-response relationship of neonatal TSH with the risk of a score below national minimum standard for numeracy and reading. A higher risk was demonstrated at concentrations when screening for congenital hypothyroidism was considered negative (the 90th percentile onwards), finally reaching a 75% and 42% higher risk, respectively. Exhibiting a similar dose-response relationship, neonatal TSH concentrations were associated with vulnerability in developmental domains or having special needs (from the 98th percentile onwards). Importantly, the risk of these outcomes did not differ for neonates with a TSH above (or near) the threshold used to further screen for congenital hypothyroidism, suggesting a positive effect of identification and treatment of children with congenital hypothyroidism.

Important caveats within the research field of early thyroid hormone exposure should also be recognized, and a combination of epidemiological and translational-physiological aspects can arise. Again, as an example, the national dataset linkage methodology used by Lain et al. to study this large number of children does come at the cost of specificity. From a methodological point of view, misclassification bias is an important issue and also the possibility of residual confounding, despite adequate adjustments for many potential confounders, cannot be excluded. From a biological and clinical perspective, the data lack description on, or clues pointing towards, the underlying pathology. However, a combination of certain biological mechanisms seem likely to contribute to the results by Lain et al.; First of all, a small group of missed neonates with congenital hypothyroidism that did not receive (timely) treatment may pull the association. This would indicate a lack of sensitivity in current screening programmes. Secondly, high neonatal TSH concentrations may reflect low maternal thyroid hormone availability and/or low maternal iodine status during early pregnancy.[15,21]

Third, borderline high TSH concentrations may represent children with a subclinical form of congenital hypothyroidism due to for example minor anatomical defects or (unknown) genetic alteratioans associated with a mild phenotype. This might result in prolonged exposure to low-normal thyroid hormone concentrations during childhood, subsequently affecting postnatal brain development.

Follow-up studies are necessary to reveal the true clinical relevance of a potential subclinical form of congenital hypothyroidism, as suggested in this study. However, beyond the complexity of the maternal-fetal interplay of thyroid hormone regulation and action in the perinatal period, the findings by Lain

and colleagues do unveil a high-risk group and their results warrant further studies. A logical next step would be to study the association of heel prick TSH concentrations with other features of congenital hypothyroidism such as hearing loss, overweight and abnormal bone maturation, and studies that asses maternal and child medication usage, using linkage to other national datasets.[22]

STUDIES ON THYROID FUNCTION REFERENCE RANGES DURING PREGNANCY

As in accordance with recommendations by the International Federation of Clinical Chemistry[23], international thyroid guidelines advise that reference ranges should be based on the 2.5th and 97.5th percentile of the respective population with an optimal iodine intake.[6-8] In addition, each study on a specific endpoint could also incorporate a sensitivity analysis on neighboring cut-off percentiles in order to explore the optimal cut-off point. Analyses using a non-parametric cut-off should be performed in a sufficiently sized, non-selected population which consists of 'healthy' reference subjects. Because of the high interperson variability and skewness of particularly TSH, but also to some extent FT4, a minimum of ~400 individual measurements per partition is required as opposed to the minimum of 120 measurements recommended for standard parametric 90% coverage interval calculations.[24-27] Although the term 'healthy subjects' can be interpreted in many ways for TSH and FT4 reference range determinations, this at least means a population free of major known thyroid function inhibiting or stimulating factors. Preferably, this population would consist of thyroid antibody (i.e., thyroid peroxidase antibody (TPOAb)) negative women without pre-existing thyroid disease or other thyroid interfering factors (such as medication use, twin pregnancies etc.). Exclusion of TSH receptor antibody (TRAb) positive subjects could further improve reference range estimations, although most of the TRAb-positive subjects are also TPOAb-positive and TRAbs are far less common in the general population than TPOAbs.[28]

Table 1 shows reference ranges for TSH and FT4 during early pregnancy calculated according to the international guidelines in sufficiently sized population-based cohorts amongst TPOAb-negative women.[9,14,29-40] For both hormones, a wide range of normal values has been reported with the upper limit of TSH varying between 2.15 and 4.68 mU/L between different cohorts. Importantly, 90% of all upper limits of TSH are higher than the recommended fixed TSH cut-off levels of 2.5 and 3.0 mU/L for the first and second trimesters, respectively. The clinical relevance of this finding is that the use of these fixed upper limits of 2.5 mU/L and 3.0 mU/L therefore results in significant over-treatment in euthyroid women which may have negative effects on maternal and/or fetal outcomes. This is illustrated in Figure 1 for a large iodine sufficient population-based cohort in The Netherlands, where 8.6 and 4.9 % of the TPOAb-negative women with normal range TSH levels had a TSH level above 2.5 mU/L and 3.0 mU/L in the first and second trimesters, respectively.[36] These data underline the importance of calculating population-based pregnancy-specific thyroid parameter reference ranges, instead of using fixed upper limits of 2.5 and 3.0 mU/L.

TABLE 1. *Reference ranges for TSH and FT4 during early pregnancy worldwide.*

			TSH in mU/L		FT4 in pmol/L (ng/dl)			Population characteristics		
Author, Country (reference) (analyzing method)	**N**	**Gestation (week)**	**Median**	**2.5th-97.5th**	**Median**	**2.5th-97.5th**	**(Median, 2.5th-97.5th)**	**Iodine insufficiency**	**Mean BMI**	**Ethnicities (%)**
Bestwick et al., Italy (19) (AutoDELFIA)	5505	<16	1.07	0.04 - 3.19	9.3	7.4 - 12.2	(0.73, 0.58 - 0.95)	Moderate-Mild	[a]	NR
Bestwick et al., UK (19) (Advia Centaur)	16,334	<16	1.11	0.06 - 3.50	13.9	10.9 - 17.9	(1.08, 0.85 - 1.40)	Moderate-Mild	[a]	NR
Bocos-Terraz et al., Spain (20) (Architect)	481	<14	0.94	0.41- 2.63	13.9	10.8 – 17.8	(1.08, 0.84 - 1.38)	Mild	NR	White (93%)
Gilbert et al., Australia (21)[b] (Architect)	1817	9-13	0.74	0.02 - 2.15	13.5	10.4 - 17.8	(1.05, 0.81 - 1.39)	Borderline	NR	Australian
Lambert-Messerlian et al., USA (22)[c] (Immulite 2000)	8351	T1	1.00	0.12 - 3.37	14.2	10.4 – 17.8	(1.10, 0.81 - 1.38)	Mild	NR	White (67) and Hispanic (23)[d]
	8415	T2	1.19	0.35 - 3.35	13.0	9.3 – 16.2	(1.01, 0.72 - 1.26)			
La'ulu et al., USA (23,24)[e]	2172	10-13	0.94	0.02 - 2.69	14.7	11.4 - 18.6	(1.15, 0.89 - 1.45)	Mild	NR	Hispanic (37), White (29), Black (27), Asian (8)
	2683	14-20	1.14	0.15 - 3.11	12.0	9.3 - 15.2	(0.94, 0.73 - 1.19)			
Li et al., China (25) (Cobas Elesys 601)	640	7-12	1.47	0.10 - 4.34	15.8	12.3 - 20.9	(1.23, 0.96 - 1.63)	Proven sufficient[f]	NR	Chinese (presumed)
Männisto et al., Finland (16) (Architect i2000)	4333	T1	1.11	0.08 – 3.54	15.3	11.7 – 22.8	(1.12, 0.86 - 1.58)	Sufficient	22.4	Finnish (presumed)
	747	T2	1.37	0.11 – 4.24	14.6	11.2 – 23.4	(1.13, 0.87 – 1.82)			
Medici et al., the Netherlands (7) (Vitros ECI)	5186	8-18	1.30	0.03 - 4.04	14.7	10.4 - 22.0	(1.15, 0.81 - 1.72)	Proven sufficient[f]	24.5	Dutch (52), Surinamese/Antillean (12), Turkish (8), Moroccan (6)
Pearce et al., USA (26) (Advia Centaur)	585	<14	1.1	0.04 - 3.60	2.1[h]	1.5 - 2.9[g]	-	Borderline	NR	White (77) and Black (10)
Quinn et al., Russia (27) (Abbott AxSYM)	380	T1	1.66	0.09- 4.67	-	-	-	Moderate	NR	Russian (presumed)
	549	T2	2.00	0.20- 4.68	-	-	-			
Springer et al., Czech Republic (28)[h] (ADVIA Centaur)	4337	9-11	1.21	0.06 - 3.67	-	-	-	Mild	NR	Caucasian (99)
Stricker et al., Switserland (29) (Architect i2000SR)	575	6-12	0.95	0.07 - 2.82	13.9	10.5 - 18.5	(1.08, 0.82 - 1.44)	Sufficient	NR	Swiss (presumed)
	528	T2	1.02	0.20 - 2.79	12.2	9.5 - 15.7	(0.95, 0.74 - 1.22)			
Vaidya et al., UK (30) (Modular E 170)	1089	<12	1.08	0.14 - 3.19	14.6	10.7 - 19.4	(1.12, 0.83 - 1.59)	Mild-moderate	NR	White (91) and South Asian (4)

Studies were selected according to the following criteria: N≥500, exclusion of TPOAb positive women and availability of data from the manuscript or via personal communication. Iodine status was estimated based on references from article, WHO iodine status reports or from the Vitamin and Mineral Nutrition Information System (VMNIS).
TSH, thyroid-stimulating hormone; FT4, free thyroxine; NR, Not reported; T1, first trimester; T2, second trimester.
a Weight reported (Bestwick et al. median weight 59 kg in Italian and 67 kg in UK population);b Reported FT4 level is a mean; c Limits are 5th and 98th percentiles for TSH and 2nd and 95th percentiles for FT4; d Based on reports of the total FASTER population; e FT4 determined in normal-range TSH only; f Based on iodine measurements in study population; g Free T4 index (normal range 1.0-4.0); h High hCG levels excluded.

FIGURE 1. *Distribution of normal range serum TSH levels in the first and second trimester in a Dutch cohort of TPOAb negative pregnant women*

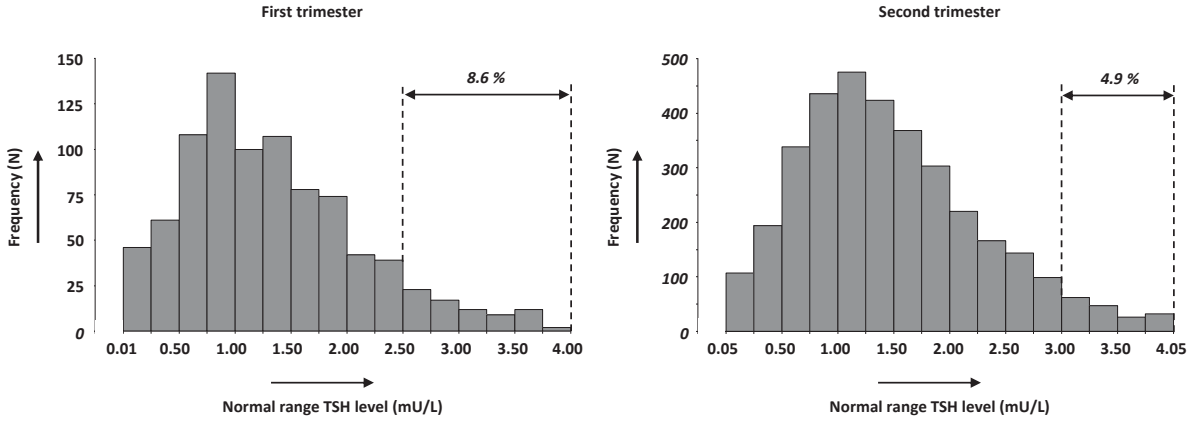


Fig.1 Adapted from Medici et al. (J Clin Endocrinol Metab. 2012; 97(2):646-52) (28). Distribution of normal range serum TSH levels (2.5th – 97.5th percentiles) in the first and second trimester in 5186 Dutch women, after exclusion of women with TPOAb positivity, known thyroid disease, thyroid (interfering) medication usage, twin pregnancies, and pregnancies after fertility treatment. In the first trimester, 8.6 % of the women with normal range TSH levels had a TSH level > 2.50 mU/L. In the second trimester, 4.9 % of the women with normal range TSH levels had a TSH level > 3.00 mU/L. TPOAb, thyroid peroxidase antibody; TSH, thyroid-stimulating hormone.

A TYPICAL CASE ILLUSTRATING THE CONSEQUENCES OF SUBOPTIMAL REFERENCE RANGE RECOMMENDATIONS

In a recent issue of JAMA Internal Medicine, Maraka and colleagues describe a clinical case of a healthy pregnant woman complaining of fatigue during early gestation (8 weeks). Thyroid function tests showed a TSH of 2.8 mU/L, leading to the diagnosis of subclinical hypothyroidism according to the recommendations of international guidelines.[41-43] These guidelines state that in absence of population-based reference ranges, a fixed upper cut-off for TSH levels of 2.5 during the first and 3.0 mU/L during the second trimesters is recommended.[2,3] In the case described, treatment with 50µg levothyroxine resulted in hyperthyroid symptoms leading to her anxiously seeking emergency care.

Overtreatment may occur more often than we think. During pregnancy, human chorionic gonadotropin (hCG) stimulates the maternal thyroid but in contrast to TSH, hCG production is not regulated via negative feedback from FT4. Therefore levothyroxine treatment during pregnancy may lead to high FT4 levels, particularly when treatment is started before the hCG peak (~10 weeks). Indeed, 10% of women treated for mild gestational thyroid dysfunction needed dose reduction in a large clinical trial.[44] Most studies focus on low thyroid function, yet high thyroid function is associated with preeclampsia and decreased birth weights,[45] indicating that more data is needed on the effects of high thyroid hormone levels and levothyroxine treatment.

As the authors point out, the proportion of women diagnosed with subclinical hypothyroidism is likely too high. We fully agree on this point, but feel that a crucial factor that could aid physicians in decision making remains under-addressed in their report.

As is shown in this introduction, since publication of the guidelines[2,3], 14 sufficiently sized studies on reference ranges have been published. Although reference ranges differ according to population characteristics such as iodine status, ethnicity and BMI, 12 of these studies (n=63.362) reported an upper trimester-specific TSH limit that was higher than 2.5 or 3.0 mU/L.[45] In addition, sensitivity analyses

show that identification of women at risk for adverse pregnancy outcomes associated with high TSH is better when using a population-based cut-off as compared to the fixed TSH cut-off levels.[45]

These data strongly suggest that a large proportion of women worldwide will be overdiagnosed with subclinical hypothyroidism when fixed upper limits of TSH of 2.5 or 3.0 mU/L are used instead of population based reference ranges. We believe that doctors without access to population-based reference ranges are more likely to correctly diagnose subclinical hypothyroidism by adapting population-based reference ranges from populations with similar demographic characteristics.

FACTORS INFLUENCING THYROID FUNCTION REFERENCE RANGES DURING PREGNANCY

As illustrated by Table 1, various commercial TSH and FT4 assays have been used to evaluate thyroid function during pregnancy. While previous studies have shown that the inter-assay differences for TSH are relatively small (r=0.91-0.98), FT4 measurements seem much more prone to interference and have larger inter-assay differences (r=0.68-0.89).[46,47] Pregnancy results in a shift of potentially interfering factors such as TBG and albumin. Not only does the extent of this shift vary per individual, but they also affect measurements by each immunoassay differently. Therefore, the population differences in FT4 levels can be at least partly attributed to assay-related factors. As recently suggested by Bestwick *et al.*, TSH and FT4 values can be expressed as multiple of medians (MoM), in order to interpret and compare the upper and lower limits obtained via different assays.[29] A MoM value is calculated by dividing each individual's value by the population median, which creates a value that is standardized for the assay median. These values are independent of inter assay differences and therefore cut-off points for different assays can be generalized more easily. Table 2 shows the calculated lower and upper limits expressed as MoM values for the same studies as Table 1, which resulted in more uniform reference ranges. This is especially the case for FT4, suggesting that TSH is more subject to change by non-analytical factors.

It has been known for long that iodine is an essential component of TH that is subject to physiologic changes during pregnancy, including an increased turnover and renal excretion, necessitating increased intake during pregnancy. It is therefore expected that populations with an abnormal iodine status have a higher prevalence of thyroid dysfunction, which would lead to unreliable reference range estimations. For this reason, the international guidelines recommend calculating reference ranges in populations with an optimal iodine intake.[6-8] Despite this, little data are available about the exact effects of iodine status on thyroid function reference ranges during pregnancy. A Chinese study recently measured first-trimester serum thyroid function and urinary iodine concentrations (UIC) in 7,190 pregnant women from an iodine-sufficient population.[48] No effects of low UIC on mean serum TSH or FT4 levels were observed. However, compared to women with adequate iodine intake (UIC 150-249 µg/L), women with excessive iodine intake (UIC>500 µg/L) had higher mean TSH (2.32 vs 1.86 mU/L) and lower FT4 (15.27 vs 16.12 pmol/L) levels (all $P<0.001$). Calculated serum TSH and FT4 reference ranges were 0.24-5.63 mU/L and 12.23-21.01 pmol/L in women with adequate iodine intake, and 0.36-6.12 mU/L and 12.14-20.64 pmol/L in women with excessive iodine intake. More studies in various trimesters of pregnancy and different ethnicities are needed to extrapolate the exact extent of these effects.

The extent to which other population characteristics such as ethnicity, BMI and smoking influence TSH or FT4 measurements is much better quantifiable. These characteristics have all been associated with differences in serum thyroid parameters as well.[29,32,33,35,37,49-56] With regard to ethnicity, it has been shown that both upper and lower limits for a wide range of serum thyroid function tests differ according to ethnic background in the first and second trimester. La'ulu *et al.* showed substantial differences in

TSH upper limits, ranging from 2.73 in blacks (MoM 2.81) to 3.64 mU/L in Asians (MoM 3.17), reaching borderline statistical significance.[32,33] Recently, we have shown significant differences in TSH reference ranges between various ethnic groups in a population-based pregnancy cohort from European origin (Supplemental Figure 1), and additionally demonstrated that these ethnic differences in thyroid parameter reference ranges may lead to considerable misclassification of thyroid disease in up to 18% of cases.[52]

TABLE 2. *Reference ranges for TSH and FT4 during early pregnancy worldwide, expressed as MoMs.*

Study	Gestational week	P2.5 - P97.5	P2.5 - P97.5
Bestwick *et al.,* UK (12)	<16	0.05 - 3.15	0.78 - 1.29
Bocos-Terraz *et al.*, Spain (13)	<14	0.44 - 2.80	0.78 - 1.28
Gilbert *et al.*, Australia (14)	9-13	0.03 - 2.91	0.77 - 1.32
Lambert-Messerlian *et al.*, USA (15)	T1	0.12 - 3.37	0.73 - 1.25
	T2	0.29 - 2.82	0.72 - 1.25
La'ulu *et al.*, USA (16,17)	10-13	0.02 - 2.86	0.78 - 1.27
	14-20	0.13 - 2.73	0.78 - 1.27
Li *et al.*, China (18)	7-12	0.07 - 2.95	0.78 - 1.32
Männisto *et al.*, Finland (19)	T1	0.07 - 3.19	0.76 - 1.49
	T2	0.08 - 3.09	0.77 - 1.60
Medici *et al.*, the Netherlands (20)	8-18	0.02 - 3.11	0.71 - 1.50
Pearce *et al.*, USA (21)	<14	0.04 - 3.27	-
Quinn *et al.*, Russia (22)	T1	0.05 - 2.81	-
	T2	0.10 - 2.34	-
Springer *et al.*, Czech Republic (23)	9-11	0.05 - 3.03	-
Stricker *et al.*, Switserland (24)	6-12	0.07 - 2.97	0.76 - 1.33
	T2	0.20 - 2.74	0.78 - 1.29
Vaidya *et al.* UK (25)	<12	0.13 - 2.95	0.73 - 1.33

P2.5-97.5, 2.5th–97.5th percentiles; TSH, thyroid-stimulating hormone; FT4, free thyroxine; MoMs, multiple of medians
a Based on iodine measurements in study population.
MoM values were calculated by dividing each individual TSH or FT4 value by the (trimester-specific) median value. These values were extracted from the original manuscript or obtained via personal communication with the study authors.

Also BMI has been associated with both TSH and FT4 levels during pregnancy.[29,35,50,54] Männisto *et al.* found that the upper limit (95th percentile [P95]) for TSH increased from 2.86 mU/L in women with a BMI <20 kg/m^2, to 3.50 mU/L amongst women with a BMI >30 kg/m^2. For the same groups, they also showed that the lower limit for FT4 (P5) decreased from 12.3 pmol/L to 11.6 pmol/L, respectively.[35] Bestwick *et al.* expressed these values in MoMs and found an increase in TSH of 0.025 MoM, and a decrease in FT4 of 0.009 MoMs per 10 kg increase in body weight.[29] In this context it is noteworthy that the prevalence of overt hypothyroidism in morbidly obese subjects (BMI>40 kg/m^2) was found to be 11.8%.[57] The guidelines of the American Thyroid Association therefore recommend TSH screening in morbidly obese pregnant women.[7]

In line with the above, Table 1 additionally shows data on BMI, iodine status, and specific ethnic backgrounds for the various studies on thyroid function reference ranges. However, it is hard to comment on these associations from this table, as these characteristics were incompletely reported in many of these studies.

Finally, various studies have shown that smoking only has limited effects on mean TSH and FT4 levels during pregnancy.[29,37,53,55,58] This is illustrated by a study amongst 4317 Finnish pregnant women which found that smokers had similar TSH levels compared to non-smokers (1.02 versus 1.02 mU/L) whereas there was a small difference in FT4 levels (15.02 versus 15.24 pmol/L; *P*=0.006).[53] As effect sizes are small, it seems unlikely that population differences in smoking prevalence have any noteworthy effect on TSH and FT4 reference ranges.

MINOR VARIATIONS IN THYROID FUNCTION AND THE RISK OF ADVERSE MATERNAL AND CHILD OUTCOMES

The previous paragraphs showed that there are substantial differences in thyroid parameter reference ranges between populations. However, what is the clinical relevance of using these population-based pregnancy-specific ranges instead of fixed or non-pregnancy ranges? In Supplemental Table 1 we calculated these effects in the Generation R study. Women with TSH levels above the population-based reference range had an increased risk of premature deliveries and children with intrauterine growth retardation (SGA; small size for gestational age), while women with TSH levels below this range had an increased risk of hypertensive disorders (references[59,60] and unpublished results). These associations disappeared when fixed TSH cut-offs were used, illustrating that women with a TSH between the fixed and population-based TSH reference range cut-offs do not have an increased risk of these complications, which suggests that the use of fixed instead of population-based reference ranges would lead to over-treatment. In recent years, various other studies have investigated the effects of minor subclinical variations in thyroid function on the risk of adverse maternal and child outcomes. These studies are important in the clinical context of this review, as they provide insight into the potential consequences of applying incorrect reference ranges to a given pregnant population. Below, we will provide an overview of the effects of subclinical thyroid dysfunction during pregnancy on the risk of a number of important and well-studied maternal and child complications, as summarized in Table 3 [59-78]. A detailed discussion of studies on overt thyroid dysfunction is beyond the scope of this review, as it has already been known for long that overt thyroid dysfunction is associated with these pregnancy complications, and differences in reference range determination particularly affect the identification of subclinical disease.

TABLE 3. *Subclinical thyroid dysfunction during pregnancy and the risk of maternal and child adverse outcomes.*

Thyroid (dys)function group	Pregnancy loss	Prematurity	Hypertensive disorders	Low birth weight
Subclinical hypothyroidism	↑ (42)	? (41,42,45-50)	↔ (47-49,51-56)	↔ (41,48-50,55,57,58)
Subclinical hyperthyroidism	? (41,43)	? (41,43,45,50)	↔ (43,51,56)	↔ (41,43,50)
Normal-range FT4 levels	? (44)	↔ (44)[a]	? (44,56)	↑ (44,59,60)[b]

References to studies on respective thyroid (dys)function group and adverse outcome are shown between brackets. FT4, free thyroxine
↑ increased risk; ↔ no effect; ? contradictory results or limited data.
[a] Only tested gestational age at birth <37 wks.
[b] High-normal FT4 levels (center-specific reference ranges) associated with lower birth weight and higher risk of SGA (small size for gestational age) newborns.

Pregnancy loss

Pregnancy loss is a difficult study endpoint because early fetal loss naturally occurs in approximately 30% of pregnancies, of which the majority occurs even before pregnancy is clinically recognized.[79] Negro

et al. studied the relationship between thyroid function and the combined endpoint of miscarriage and stillbirth in TPOAb-negative pregnant women and concluded that women with serum TSH levels of 2.5-5.0 mU/L had a 6.1% risk of pregnancy loss, compared to 3.6% in women with a TSH level below 2.5 mU/L.[62] However, the fact that no population-based reference ranges were calculated or sensitivity analyses were done, makes the 2.5 mU/L cut-off somewhat arbitrary and hard to interpret in relation to other studies. Further analyses showed a positive linear association between TSH levels and pregnancy loss. This is in line with the results of a Dutch cohort of 2497 pregnant women, in which it was shown that the incidence of miscarriage, fetal and neonatal death (combined into child loss) increased by 80% by every doubling of the maternal TSH concentration.[80] However, given the limited number of 27 cases and the heterogeneity of cases included in this group, these results should be interpreted with caution. Ashoor *et al.* retrospectively measured thyroid parameters in early-pregnancy samples taken from 202 pregnancies that would subsequently end in miscarriage or fetal loss and 3592 normal pregnancies.[81] Although the associations with subclinical thyroid dysfunction were not formally tested (i.e., abnormal TSH with still normal FT4), the pregnancies complicated by child loss had higher mean TSH and lower FT4 levels, and a higher prevalence of TSH levels > P97.5 and FT4 levels < P2.5. Finally, early-pregnancy TSH levels >P95 were associated with an increased risk of miscarriages (OR 3.66, *P* = 0.002) in an Australian pregnancy cohort, although subclinical and overt hypothyroid cases were pooled.[82] Taken together, these studies do suggest an increased risk of pregnancy loss in pregnancies with subclinical hypothyroidism, but large prospective studies from conception onwards are needed to determine the exact magnitude of effects.

Premature delivery

Premature delivery is the leading direct cause of child death in almost all high- and middle- income countries and is associated with substantial morbidity later in life.[83-85] Subclinical hypothyroidism has been described as a risk factor for premature deliveries, although the pathophysiological mechanism remains poorly understood. The largest study on this association has been performed by Casey *et al.* in a cohort of 17,298 pregnant women presenting for prenatal care.[66] Subclinical hypothyroidism (TSH >P97.5 and normal range FT4) was associated with a slightly increased risk of prematurity < 34 wks (4% vs 2.5%, *P*=0.01), borderline significantly associated with prematurity <32 wks (2.5 vs 1%, *P*=0.07) and not associated with prematurity <36 wks (7 vs 6%, *P*=0.39). This is in line with a later study by Cleary-Goldman *et al.* showing that subclinical hypothyroidism (TSH >P97.5 and normal range FT4) was not associated with prematurity <37 wks, whereas the effects on earlier premature deliveries were not investigated.[67] Various other studies have also investigated these relations, with conflicting results.[61,62,65,68,69,71,82] This can be partly explained by the fact that some studies pooled overt and subclinical hypothyroid cases[71,82], some included a limited number of premature deliveries[68,69], while others used different TSH cut-off values.[62,65] We therefore studied the association between increased TSH levels and the risk of premature deliveries using a population-based P97.5 (4.0 mU/L) and a fixed 2.5 mU/L cut-off.[59] While no associations were seen with a TSH >2.5 mU/L, a 1.9 and 2.5 times increased risk of prematurity <37 and <34 wks was seen among women with a TSH >4.0 mU/L. However, this association no longer persisted after exclusion of TPOAb-positive women or women with comorbidities. This shows that these factors confound the observed associations and underlines the importance of performing in-depth analyses in a detailed cohort, taking the interfering role of various confounders into account.

Far less data are available on the effects of subclinical hyperthyroidism on prematurity. In a study in women presenting for prenatal care, subclinical hyperthyroidism (n=433) was not associated with prematurity ≤36, ≤34 and ≤32 weeks.[63] This is in line with a population-based cohort study by Mannisto

et al. in which subclinical hyperthyroidism (n= 224 cases) was not associated with prematurity <37 and <34 weeks either.[61] While two other population-based studies also did not find any associations, it should be noted that their analyses were limited by a small number of subclinically hyperthyroid cases (n=77 and 31).[59,69]

Hypertensive disorders

Hypertensive disorders, including gestational hypertension and (pre)eclampsia, are common during pregnancy and are an important cause of maternal and fetal morbidity and mortality.[86,87] Both hypo- and hyperthyroidism have vascular effects, including endothelial cell dysfunction[88,89], and are associated with an increased risk of hypertensive disorders during pregnancy. Therefore, many studies have also investigated the effects of subclinical thyroid dysfunction on the risk of hypertensive disorders. Although a part was limited by their small number of subclinically hypothyroid or hypertensive cases[60,68,73,74], a few of these studies were carried out in large pregnancy cohorts. [66,67,70-72] In a prospective cohort study in nearly 25,000 pregnancies by Wilson *et al.*, subclinical hypothyroidism (TSH>P97.5 and FT4 P2.5-97.5) was associated with a 1.6-fold increased risk of severe preeclampsia.[72] However, the fact that this association disappeared when only women screened before 20 weeks of gestation were included is suggestive of reverse causality.[66] This could be due to for example placental factors that are increased in preeclampsia and affect thyroid function.[90] Indeed, the other large studies did not find a relation between subclinical hypothyroidism in early-pregnancy and the risk of subsequent hypertensive disorders.[67,70,71] The previously mentioned study by Wilson *et al.* also studied subclinical hyperthyroids and did not find any effects either[72], as replicated in Finnish and Dutch population-based cohorts.[60,70] Whereas the latter cohort was limited by the small number of subclinically hyperthyroid cases (n=62), it also investigated the effects of variation in thyroid function within the population-based calculated P2.5-97.5 ranges, and found an increased risk of preeclampsia in pregnancies with high-normal FT4 levels.[60] In contrast, a decreased risk of preeclampsia in pregnancies with high-normal FT4 levels was detected in a recent study by Haddow *et al.*, although these effects were borderline significant and *P*-values had not been corrected for multiple-testing.[64] Therefore, future studies will have to clarify if even variation in FT4 levels within population-specific reference ranges affects the risk of hypertensive disorders during pregnancy.

Low birth weight

A low birth weight can either be due to SGA or prematurity, and has been associated with an increased risk of perinatal morbidity and mortality.[91,92] The previously mentioned study by Cleary-Goldman *et al.* was the first large study to investigate the relationship between subclinical hypothyroidism and birth weight, and showed no effect on the risk of newborns with very low (<2500 g) or high (>4000 g) birth weights.[67] A subsequent study by Mannisto *et al.* investigated these relations with both subclinical hypo- and hyperthyroidism in more detail, and did not find effects on the risk of SGA or large size for gestational age newborns either, while it also showed no differences in mean birth weights between these groups.[61] A few other studies have investigated these relations, with conflicting results, which is likely to be due to their substantially smaller sample sizes.[68,69,74-76] As opposed to studying subclinical thyroid dysfunction groups, Shields *et al.* were the first to study the relation between continuous FT4 levels and birth weight in a population-based cohort after excluding women with overt thyroid dysfunction, and found a statistically significant negative relation between FT4 and birth weight.[78] These relations have been subsequently studied in Dutch pregnant women with FT4 levels within their center-specific reference ranges, showing that high-normal FT4 levels are not only associated with lower mean birth weights, but also with more SGA and <2500 g newborns.[77] These results have recently been

convincingly replicated in the recent study by Haddow *et al.*, which additionally showed that these children do not suffer from more labor/delivery complications.[64] As a low birth weight is a risk factor for cardiovascular and psychiatric diseases in later life[91,93], it would be interesting to follow these children up for the occurrence of these complications.

CONCLUSIONS

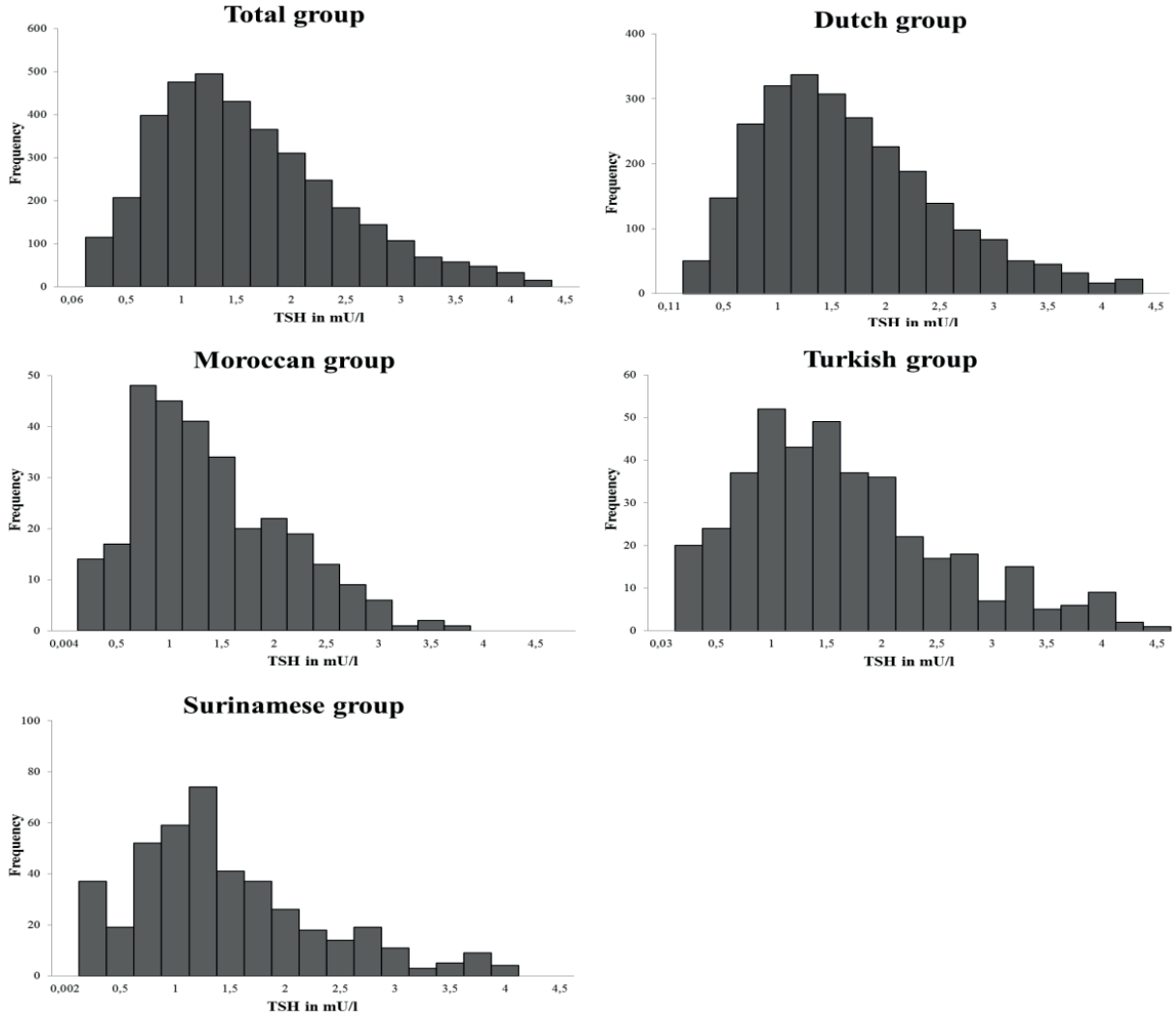
In the last decade a large number of studies have been published on thyroid function reference ranges during pregnancy. In the current review we show that there are large differences in TSH and FT4 reference ranges between these populations, with 90% of these studies having higher upper limits of TSH than the fixed TSH cut-off levels of 2.5 and 3.0 mU/L that are currently advocated in the guidelines.[6-8] Nevertheless, most institutions still rely on the fixed TSH cut-off levels of 2.5 and 3.0 mU/L for the first and second trimesters respectively.

The use of MoMs illustrated that part of the differences in these ranges between populations can be explained by the use of different assays, while also a number of population-specific characteristics such as ethnicity and BMI have been identified as determinants of reference ranges. Provided that institutions determine their own population-based ranges, there is no direct need for using MoMs in clinical practice. However, the universal use of MoMs in clinical studies on the effects of thyroid dysfunction during pregnancy would certainly be useful, since it will facilitate comparison and meta-analysis of results.

We therefore conclude that institutions should not rely on a fixed universal cut-off level throughout the world, but should calculate their own pregnancy-specific population-based ranges. If such reference ranges are not available, adopting population-based reference ranges from a population with similar characteristics would be the best option.

APPENDIX

SUPPLEMENTAL FIGURE 1. *Distribution of normal range TSH levels in the total group of pregnant women from the Generation R Study, as well as for the different ethnic subgroups separately.*



Supplemental Fig. 1. Adapted from Korevaar et al. (J Clin Endocrinol Metab. 2013; 98(9):3678-3686) (39). Histograms showing the distribution of normal range TSH values for all 3944 women with available serum thyroid parameters from the Generation R Study, as well as for the different ethnic subgroups separately. Normal ranges for TSH were defined as the 2.5-97.5th percentiles of the respective group after exclusion of pre-existing thyroid disease, thyroid (interfering) medication use, fertility treatment, twin pregnancies and TPOAb-positive women.

1

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PART 1: DETERMINANTS OF MATERNAL THYROID FUNCTION DURING PREGNANCY

CHAPTER 2

ETHNIC DIFFERENCES IN MATERNAL THYROID PARAMETERS DURING PREGNANCY

Tim I.M. Korevaar*, Marco Medici*, Yolanda B. de Rijke, Willy Visser, Sabine M.P.F de Muinck Keizer-Schrama, Vincent V.W. Jaddoe, Albert Hofman, H. Alec Ross, W. Edward Visser, Herbert Hooijkaas, Eric A.P. Steegers, Henning Tiemeier, Jacoba J. Bongers-Schokking, Theo J. Visser and Robin P. Peeters

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ABSTRACT

CONTEXT Abnormal maternal thyroid function during pregnancy is associated with various complications. International guidelines advocate the use of population-based trimester-specific reference ranges for thyroid function tests. When unavailable, an upper TSH limit of 2.5 for the first,- and 3.0 mU/L for the second and third trimesters is recommended. Although inter-individual differences in thyroid function tests can partially be explained by ethnicity, data on the influence of ethnicity on TSH and (F)T4 reference ranges during pregnancy are sparse.

DESIGN Serum TSH, FT4, T4, and TPOAb levels were determined during early pregnancy in 3944 women from the Generation R study, Rotterdam, the Netherlands.

RESULTS The study population consisted of 2765 Dutch, 308 Moroccan, 421 Turkish and 450 Surinamese women. Mean TSH was higher in Dutch and Turkish women than in Moroccan or Surinamese women (1.50-1.48 *vs.* 1.29-1.33 mU/L;*P*<0.01). Although no differences in FT4 were seen, T4 was lowest in Dutch women (142 *vs.* 150-156 nmol/L;*P*<0.01). Turkish women had the highest frequency of TPOAb positivity (9.3% *vs*. 5.0-5.8%;*P*<0.05) and of elevated TSH levels in the second trimester (11.0% *vs*. 3.8-7.3%;*P*<0.01). A comparison of disease prevalence between a population-based versus an ethnicity-specific reference range changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test.

CONCLUSIONS We show ethnic differences in serum TSH, T4 and TPOAb positivity and found significant diagnostic discrepancies depending on whether population or ethnicity-specific reference ranges were used to diagnose thyroid disease.

INTRODUCTION

Abnormal maternal thyroid function during pregnancy is associated with various maternal and child complications such as preeclampsia, miscarriage, preterm delivery and impaired neurodevelopment of the child.[1-3] Recent guidelines by the Endocrine Society and the American Thyroid Association (ATA) advocate the use of population-based trimester-specific reference ranges to diagnose thyroid dysfunction in pregnant women. When trimester-specific reference ranges are not available in the laboratory, upper TSH limits of 2.5 mU/L during the first,- and 3.0 mU/L during the second and third trimesters are recommended.[4,5] These recommendations are mainly based on large studies conducted in divergent populations from America, Europe, China and India which can nowadays be considered multi-ethnic as a result of increased migration.[6-11] A number of studies have shown that inter-individual differences in thyroid hormone levels may, at least partially, be explained by ethnic background.[12-14] So even within trimester-specific reference ranges, differences may exist as a result of multi-ethnicity.

Only a few studies analyzed the effect of ethnicity on thyroid function tests during pregnancy. A small study in 589 pregnant women demonstrated that African-American women have lower TSH values than Caucasian women.[15] Subsequently, La'ulu *et al.* reported that reference range values for thyroid parameters may differ between Asian, white, black and Hispanic Americans.[16,17] Benhadi *et al*. demonstrated significantly lower mean TSH levels in pregnant Dutch women compared to Turkish, Moroccan and Surinamese pregnant women.[18] In contrast, a study by Pearce *et al.* showed that ethnicity was not a contributing factor to either TSH, FT4 or T4 in pregnancy,[19] but this may have been due to the number of groups compared and a relatively small sample size.

These ethnic differences between different pregnant populations underline the importance of calculating population-specific reference ranges during pregnancy, and suggest that it may not be optimal to apply the same upper limit for TSH during pregnancy for various populations world-wide. Since most populations nowadays are considered multi-ethnic, we investigated the consequences of calculating ethnicity-specific reference ranges for the diagnosis of thyroid disease in a large, multi-ethnic population of pregnant women from Rotterdam, the Netherlands. To exclude an interfering role for iodine deficiency in specific ethnic groups, we also analyzed urinary iodine levels in a subset of pregnant women.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based cohort from early fetal life onwards in the multi-ethnic city of Rotterdam, The Netherlands, which has been described in detail previously.[20] Written informed consent was obtained from all adult participants.

Population for analyses

Data on TSH, FT4 and T4 were available for 4192, and TPOAbs for 3928 Dutch, Moroccan, Turkish and Surinamese pregnant women. Women with twin pregnancies (N=128), pre-existing thyroid disease (N=35), thyroid (interfering) medication usage (N=32) or fertility treatment (N=53) were excluded. Data on ethnicity and ethnic origin were derived by questionnaires. Ethnicity was determined by country of origin which was defined according to the classification of Statistics Netherlands[20], ethnic origin was determined by common ancestry. The final population comprised 3944 women which were included in one or more analyses.

Thyroid parameters

Maternal serum samples were obtained in early pregnancy (mean 13.4 weeks; SD 2.0). Plain tubes were centrifuged and serum was stored at -80 C. TSH, FT4 and T4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <5.4% for FT4 at a range of 14.3-25.0 pmol/L, and <6.4% for T4 at a range of 94-151 nmol/L. For TSH specifically the intra- and interassay coefficients of variation were <4.1% at a range of 3.97-22.70 mU/L, performance characteristics and comparison to other assays have been described previously.[21]

During pregnancy, profound changes in thyroid physiology occur.[4,5] Maternal supply of TH to the fetoplacental unit necessitates an increased TH production, requiring an intact thyroid gland and an adequate availability of dietary iodine. This process is in part mediated by the pregnancy hormone human chorionic gonadotrophin (hCG), which is a weak agonist of the TSH receptor and so stimulates the maternal thyroid to produce more TH.[22,23] As a consequence, reference ranges during pregnancy are different compared to a non-pregnant state.[4,5] Therefore reference ranges for TSH, FT4 and T4 were calculated for this specific population.[7] Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when greater than 60 IU/ml.[24]

Iodine measurements

Urinary iodine concentrations were determined in a random subset of 793 women during early pregnancy (mean = 12.9 weeks; SD = 1.8). Urinary iodine was measured through the ceric-arsenite reaction following destruction by means of ammonium persulphate, which has been described previously.[25]

Covariates

Information on maternal age, smoking status and socio-economic status (SES) were obtained by questionnaires during pregnancy. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI).[26]

Statistical analyses

Descriptive characteristics were compared using ANOVA, logistic regression analyses, and the Wilcoxon–Mann–Whitney U test. Total and subgroup reference ranges for maternal TSH, FT4 and T4 were defined as the range between the 2.5th and 97.5th percentiles. Additionally, TPOAb-positive women were excluded. To achieve normal distribution, TSH was logarithmically transformed. Mean TSH, FT4 and T4 levels, and TPOAb positivity were compared using ANOVA and logistic regression and additionally adjusted for maternal age, gestational age at sampling, SES, smoking, parity and BMI. The percentage of women with a TSH >2.5 mU/L in the first trimester or >3.0 mU/L in the second and third trimesters per ethnic group were compared using logistic regression analyses. The prevalences of (subclinical) hyperthyroidism, (subclinical) hypothyroidism and hypothyroxinemia in the four ethnic groups were calculated using both total-population and ethnicity-specific reference ranges.

Hyperthyroidism was defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentile) FT4; hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4 and hypothyroxinemia as a low FT4 with a normal TSH.

For pregnant populations, the WHO regards median urinary iodine levels of <150 µg/L as insufficient, 150 – 249 µg/L as adequate, 250 – 499 above requirements and >500 µg/L as excessive [20]. Median urinary iodine levels were compared between the ethnic groups using the Wilcoxon–Mann–Whitney U test. The percentage of women with urinary iodine levels <150 µg/L and >500 µg/L were compared using chi square tests and logistic regression analyses.

RESULTS

The study population consisted of 3944 women of which 70.1% were Dutch, 7.8% were Moroccan, 10.7% were Turkish and 11.4% were Surinamese. Descriptive characteristics of the studied ethnic groups are shown in Table 1. Dutch women were older, had a lower gestational age at blood sampling, had fewer pregnancies, a higher SES and lower body mass index (BMI). The Turkish women had the highest smoking prevalence.

TABLE 1 *Descriptive statistics.*

	Total population	Dutch	Moroccan	Turkish	Surinamese	P-value
Women included N (%)	3944 (100)	2765 (70.1)	308 (7.8)	421 (10.7)	450 (11.4)	
Age in years mean (SD)	30.0 (4.9)	31.1 (4.3)	28.0 (5.4)*	26.7 (4.6)*	27.6 (5.6)*	<0.01
Gestational age at sampling mean (SD)	13.4 (2.0)	13.2 (1.9)	14.3 (2.1)*	13.8 (2.1)*	13.6 (2.1)*	<0.01
Body mass index mean (SD)	24.5 (4.4)	24.1 (4.0)	26.1 (4.6)*	25.7 (5.0)*	24.8 (5.0)*	<0.01
Median TSH mU/L	1.35	1.41	1.14*	1.39	1.13*	<0.01
Median FT4 pmol/L	14.9	14.9	14.4*	14.5*	14.9	0.02
Median T4 nmol/L	144	140	151*	157*	153*	<0.01
TPOAb positivity N (%)	224 (6.1)	151 (5.8)	14 (5.0)	36 (9.3)*	23 (5.6)	0.05
Parity N (%)						
0	2271 (57.7)	1681 (60.9)	119 (38.8)*	208 (49.4)*	263 (58.4)	<0.01
1	1195 (30.4)	826 (29.9)	104 (33.9)	131 (31.1)	134 (29.8)	0.54
>1	469 (11.9)	251 (9.1)	84 (27.4)*	82 (19.5)*	52 (11.6)*	<0.01
Smoking during pregnancy N (%)						
Yes	632 (17.5)	410 (16.2)	17 (6.0)*	129 (33.9)*	76 (18.1)	<0.01
Stopped	336 (9.3)	246 (9.7)	5 (1.8)*	32 (8.4)	53 (12.6)	<0.01
Non-smokers	2645 (73.2)	1875 (74.1)	259 (92.2)*	220 (57.7)*	291 (69.3)*	<0.01
Socio-economic status N (%)						
Low	345 (8.9)	100 (3.6)	84 (29.0)*	121 (30.1)*	40 (9.0)*	<0.01
Middle	1745 (44.9)	1028 (37.4)	166 (57.2)*	216 (53.7)*	335 (75.3)*	<0.01
High	1798 (46.2)	1623 (59.0)	40 (13.8)*	65 (16.2)*	70 (15.7)*	<0.01

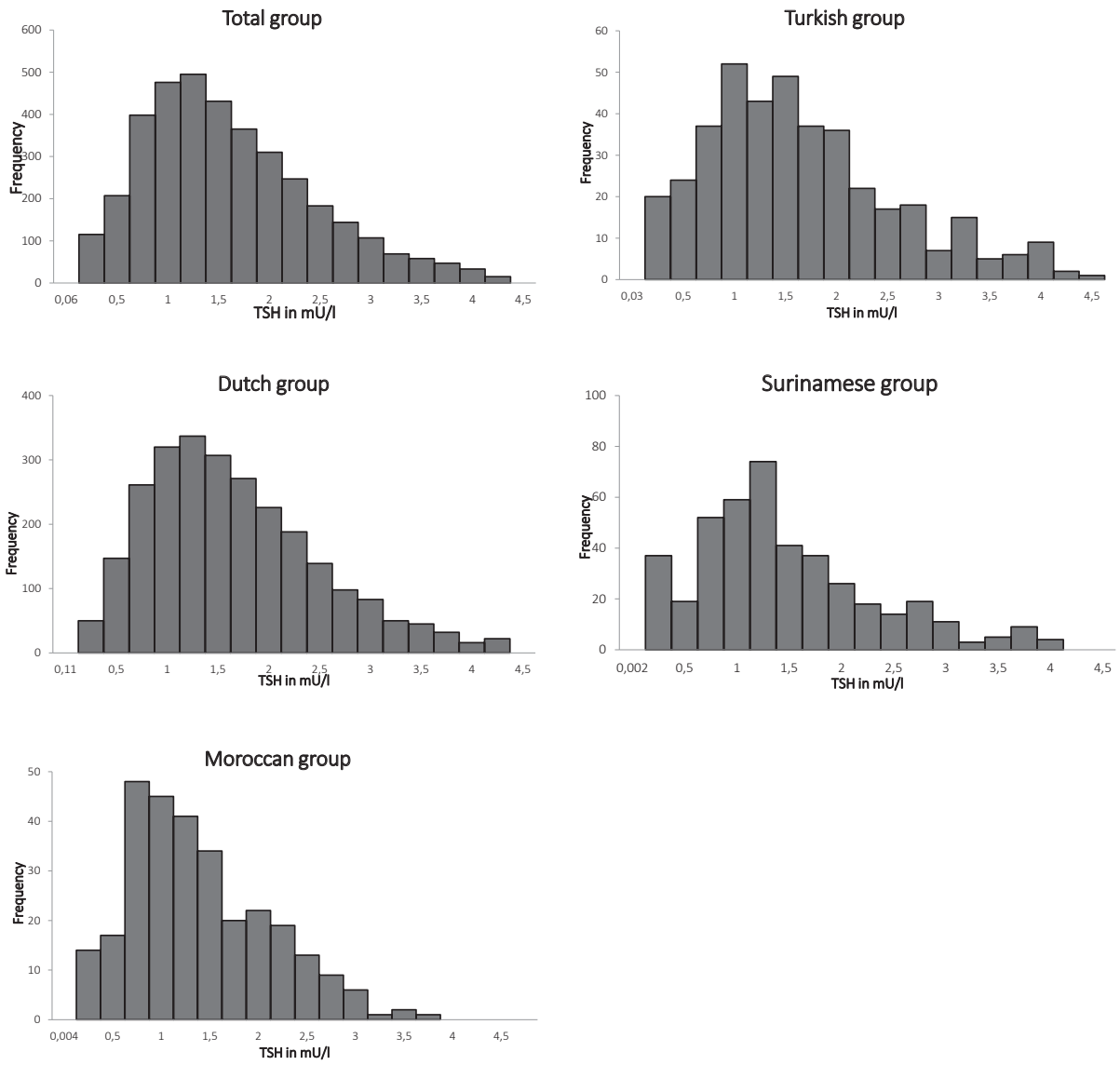
** Statistically significant (P<0.05) compared to Dutch group.*
P-values for maternal age, gestational age at sampling and BMI were calculated using ANOVA. P-values for parity, smoking, socio-economic status and TPOAb positivity were calculated using logistic regression. P-values for median thyroid hormone levels were calculated using the Wilcoxon–Mann–Whitney U test.

Ethnic differences in serum TSH, (F)T4, and TPOAb positivity.

With regard to unadjusted thyroid parameters, TSH values were significantly higher in Dutch and Turkish women than in Moroccan and Surinamese women (1.41-1.39 *vs.* 1.14-1.13 mU/L; *P*<0.01). Although unadjusted FT4 levels were significantly lower in Moroccan and Turkish women (14.4-14.5 *vs*. 14.9

pmol/L; *P*=0.02), significance was lost after correction for gestational age at sampling and exclusion of TPOAb-positive women (Table 2). T4 levels in the Dutch women were lower than those in all other ethnic groups (140 nmol/L *vs.* 151-157 nmol/L; *P*<0.01. Turkish women were more frequently TPOAb-positive compared to the Dutch women (9.3% *vs*. 5.8%; *P*<0.01). As shown in Table 2, differences in TSH and T4 between ethnic groups remained significant after exclusion of TPOAb-positive women, and after additional adjustment for maternal age, gestational age at sampling, parity, smoking, SES and BMI. Supplemental Figure 1 shows the distribution of serum TSH levels in the total population and in the different ethnic subgroups separately.

FIGURE 1. *Histograms showing the distribution of normal range maternal TSH for the total group and the separate ethnic groups.*



Normal ranges for maternal TSH were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage, fertility treatment and TPOAb positive women.

TABLE 2 *Ethnicity specific mean TSH, FT4, and T4 levels and reference ranges during pregnancy.*

	Total population	Dutch	Moroccan	Turkish	Surinamese	*P*-value[a]	Adjusted *P*-value[b]
Adjusted mean TSH (mU/L)	1.40	1.50	1.29*	1.48	1.33*	<0.01	<0.01
Reference range	0.06 – 4.51	0.12 – 4.72	0.004 – 3.99	0.04 – 4.50	0.002 – 3.85		
(TPOAb-positive excluded)	(0.06 – 4.08)	(0.11 – 4.18)	(0.004 – 3.56)	(0.03 – 4.26)	(0.002 – 3.80)		
Adjusted mean FT4 (pmol/L)	15.1	15.1	14.9	15.1	15.2	0.30	0.12
Reference range	10.4 – 21.9	10.6 – 21.8	9.9 – 21.2	9.8 – 22.5	10.2 – 23.2		
(TPOAb-positive excluded)	(10.6 – 21.9)	(10.8 – 21.8)	(9.9 – 21.0)	(9.8 – 22.3)	(10.3 – 23.9)		
Adjusted mean T4 (nmol/L)	150	142	150*	156*	152*	<0.01	<0.01
Reference range	96 – 219	95 – 204	98 – 233	105 – 242	93 – 238		
(TPOAb-positive excluded)	(96 – 219)	(96 – 204)	(97 – 231)	(104 – 238)	(93 – 246)		

* Statistically significant (*P*<0.05*)* compared to Dutch group. [a] Adjusted for gestational age at sampling. [b] Adjusted for gestational age at sampling, maternal age, SES, smoking, parity and BMI. Mean values were calculated as the mean for the 2.5th - 97.5th percentiles of TSH, TT4 or FT4 after exclusion of TPOAb positive women and after correction for gestational age. Adjusted *P*-values were additionally corrected for maternal age, parity, smoking, and socio-economic status.
Reference ranges were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage or fertility treatment, additionally TPOAb positive women were excluded.

For completeness, women from Morocco and Surinam were subsequently classified according to ethnic origin, since inhabitants of these countries belong to two or more large ethnic groups. Moroccan women were classified as Berber, Arabic or unspecified origin whereas Surinamese women were classified as Creoles, Hindustani or other origin. Analyses showed that, in addition to ethnicity (defined by country of origin), thyroid parameters may also differ according to ethnic origin (defined by common ancestry). Besides common geography, cultural habits and recent heritage, subdivision according to ethnic origin may reflect genetic similarities more thoroughly (see supplemental Table 1).

Ethnic differences in the risk of elevated TSH levels.

Current Endocrine Society and ATA guidelines recommend to use an upper limit of TSH of 2.5 mU/L in the first trimester and of 3.0 mU/L in the second and third trimesters when population-based trimester-specific reference ranges are not available. Table 3 displays the number of women with elevated trimester-specific TSH levels according to these cut-off values. Turkish women had a significantly higher frequency of elevated TSH values in the second trimester than the Dutch women (13.6% *vs*. 9.5%; *P*=0.02) whereas Moroccan and Surinamese women displayed a significantly lower frequency compared to the Dutch women (5.0-5.8% *vs*. 9.5%; *P*=0.02). This effect remained significant after the exclusion of TPOAb-positive women. Moroccan women had a borderline significantly lower frequency of elevated TSH levels (*P*=0.05).

Diagnostic consequences of the use of ethnicity-specific reference ranges.

Subsequently, we studied whether the diagnosis of (subclinical) thyroid disease in these different ethnic groups was influenced by the use of reference ranges based on the total population (total population reference range, TPRR), or based on each ethnic group separately (ethnicity specific reference ranges, ESRR). In total, of all 279 women who were diagnosed as having an abnormal thyroid function test when a TPRR was used, 51 women (18%) were re-classified when ESRR were used; 44 changed to a normal thyroid function test and 7 changed to a different disease entity. *Vice versa*, of all 3665 women who had a normal thyroid function test using TPRR, 45 (1.2%) had an abnormal thyroid function test when using ESRR. Table 4 shows the diagnostic changes per disease entity for the total group.

Iodine status in ethnic subgroups.

To exclude that the differences between different ethnic groups in our study were due to iodine deficiency in specific populations, urinary iodine levels were measured in a random selection of the total population. As is illustrated in Table 5, all ethnic groups were iodine sufficient according to the WHO criteria [20], with median urinary iodine levels between 201 and 305 µg/L. These results remained similar after adjustment for urinary creatinine (data not shown). Compared to the Dutch women, median iodine levels were significantly higher in Moroccan, Turkish and Surinamese women (201 *vs.* 235-305 µg/L) while Dutch women more often presented with urinary iodine levels <150 µg/L and less frequently with urinary iodine levels >500 µg/L.

TABLE 3 *Percentage of women with a TSH level >2.5 mU/L in the first and >3.0 mU/L in the second trimester.*

		Total population	Dutch	Moroccan	Turkish	Surinamese	*P*-value
TSH >2.5 mU/L 1st trimester	N (%)	122 (14.8)	96 (15.5)	1 (2.7)	8 (10.8)	17 (17.9)	0.17
TPOAb positive women excluded	N (%)	88 (12.0)	70 (12.6)	1 (3.0)	6 (9.5)	11 (13.6)	0.43
TSH >3.0 mU/L 2nd trimester	N (%)	278 (9.2)	199 (9.5)	13 (5.0)*	46 (13.6)*	20 (5.8)*	<0.01
TPOAb positive women excluded	N (%)	192 (7.1)	138 (7.3)	9 (3.8)	32 (11.0)*	13 (4.2)*	<0.01

** Statistically significant (P<0.05) compared to Dutch group.*
P-values were calculated using logistic regression.

TABLE 4 *Number of pregnant women diagnosed with (subclinical) thyroid disease when using the total population- (TPRR) or ethnicity specific (ESRR) reference ranges in the total group.*

	Number of subjects N (%)			
Diagnosis	**TPRR**	**ESRR**	**Change – out**	**Change – in**
Hypothyroidism	12 (0.3)	9 (0.2)	4 (36)	0 (0)
Subclinical hypothyroidism	86 (2.2)	88 (2.2)	11 (13)	11 (0.4)
Hypothyroxinemia	85 (2.2)	88 (2.2)	17 (22)	17 (0.6)
Hyperthyroidism	36 (0.9)	35 (0.9)	5 (15)	4 (0.1)
Subclinical hyperthyroidism	60 (1.5)	60 (1.5)	14 (21)	13 (0.4)
Total	**279 (7.1)**	**280 (7.1)**	**51 (18)**	**45 (1.2)**

"Change - out" is the number of pregnant women who were originally diagnosed with an abnormal thyroid function test using TPRR, but who became euthyroid or were diagnosed with a different disease entity when ESRR were used. "Change - in" is the number of pregnant women who were euthyroid when the TPRR was used, but were classified within the respective disease entity when the ESRR was used. Disease entities were diagnosed according to the reference ranges including TPOAb positive women as displayed in Table 2.

TABLE 5 *Urinary iodine levels in the 4 ethnic subgroups.*

	Total N=793	Dutch N=545	Moroccan N=76	Turkish N=90	Surinamese N=82	*P*-value
Median urinary iodine (µg/L, inter quartile range)	224 (127 – 358)	201 (109 – 329)	305* (201 – 506)	269* (178 – 368)	235* (148 – 417)	<0.01
Urinary iodine <150 µg/L (%)	239 (30.1)	193 (35.4)	11 (14.5)*	15 (16.7)*	20 (24.4)*	<0.01
Urinary iodine >500 µg/L (%)	94 (11.9)	48 (8.8)	19 (25.0)*	14 (15.6)*	13 (15.9)	<0.01

** Statistically significant (P<0.05) compared to Dutch group.*

DISCUSSION

Ethnic differences are currently not taken into account for the diagnosis of thyroid disease during pregnancy. In the current study we demonstrate that ethnic differences, even within one geographical area, may influence the diagnosis of thyroid disease. The use of ESRR instead of TPRR changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test.

Differences in TSH between pregnant women from different ethnic groups have been shown in a few other studies.[15-18] Studies in relatively small populations from different parts of the United States have shown ethnic TSH differences, without corresponding effects on FT4.[15-17,19] A study amongst 589 pregnant women found that African-American women had a median TSH value of 1.1 mU/L compared to 1.5 mU/L in Caucasian women.[15] A subsequent study amongst 2568 pregnant women in the first trimester of pregnancy found that black women had a median TSH value of 0.82 mU/L whereas white women had a median TSH level of 1.02 mU/L.[16] The same authors showed similar differences in median TSH between black and white women during the second trimester (0.97 and 1.21 mU/L, respectively). Benhadi *et al.* found that Dutch women had a higher mean TSH value than Moroccan, Surinamese and Turkish women (1.19 *vs.* 0.87, 0.91 and 0.96 mU/L respectively). Even though the study of Benhadi *et al.* was conducted in a similar population, TSH values in our study were slightly higher overall, which may be explained by different assays used to determine TSH and FT4 levels. Additional adjustment for SES in our study combined with possible differences in population iodine status, which was not assessed in the study by Benhadi *et al*., may also underlie these findings. Similar study differences may also explain why TSH levels in our study are not different between Dutch and Turkish women, despite the larger sample size in our study.

Although no significant differences in FT4 levels were observed between the different ethnic groups, T4 levels were ethnicity dependent. Data on ethnic T4 differences are sparse. A previous study in a relatively small first trimester pregnancy population by Pearce *et al*. (N=668) showed that ethnicity was not a factor significantly contributing to T4 levels.[19] However, Aoki *et al*. (N=4392) showed that T4 levels were higher in Mexican Americans compared to non-Hispanic black and non-Hispanic white Americans, but this was studied in a predominantly non-pregnant population.[27] In the current study, we found that pregnant Dutch women had significantly lower T4 levels than all other ethnic groups. The discrepancy between FT4 and T4 levels might reflect ethnic differences in binding proteins such as thyroid hormone binding globulin (TBG), transthyretin (TTR) and albumin.

In our study 224 (6.1%) women were TPOAb-positive, which is similar to what has been shown previously in other international studies and in a different pregnant populations in the Netherlands.[18,22,28] Ethnic variety of TPOAb positivity has been shown in large American studies amongst men and non-pregnant women[13], as well as in pregnant women.[16,17] However, other studies on pregnant populations failed to replicate these results.[15,18,19] Turkish women in our study had the highest prevalence of TPOAb positivity. Interestingly, Turkish women in our cohort were also more likely to smoke. Since smoking has been shown to reduce the chance of TPOAb positivity[29], it may well be that the reported increased risk of TPOAb positivity in Turkish women in this population is even an underestimation. TPOAb positivity during pregnancy is associated with an increased risk of postpartum thyroiditis, miscarriage and fetal death.[30,31] Whether Turkish women in the Netherlands are more susceptible to these pregnancy adversities remains to be investigated in future studies.

To investigate if part of the ethnic differences could be explained by differences in iodine intake, we analyzed urinary iodine excretion in a random sample of this population. As iodine intake is highly variable within populations, even iodine sufficient populations such as the United States of America and the Netherlands can contain subgroups with iodine deficiency or excess. Nevertheless, all four

ethnic groups were iodine sufficient according to the WHO criteria.[20] Compared to the other groups, the Dutch group more frequently exhibited a low urinary iodine (<150 µg/L) and less frequently a high urinary iodine (>500µg/L). However, since all populations were iodine sufficient, it is unlikely that these differences may have caused the differences in serum thyroid function tests. Furthermore, additional adjustment for urinary iodine excretion in the subset of 793 women that had this data available did not alter ethnic group differences or mean thyroid hormone levels.

In the absence of trimester-specific population-based reference ranges, TSH limits of 2.5 mU/L in the first, and 3.0 mU/L in the second trimester are recommended as trimester-specific upper limits.[4,5] Even in TPOAb-negative women, a TSH level above these cut-offs has been related to increased pregnancy loss[32], but ethnic differences high TSH levels according to these limits have not yet been investigated. Our results demonstrate ethnic differences in both the first and second trimester, with Turkish women having a higher risk of an elevated TSH than Dutch women, regardless of TPOAb status. We show that the Dutch and Surinamese women less frequently had elevated TSH levels whereas the Moroccan and Turkish women more frequently had high TSH levels in the second compared to the first trimester. Since this cannot be attributed to large ethnic differences in TSH distributions as is shown in Supplemental Figure 1, the current study does not provide an explanation for this phenomenon. We also demonstrate that the use of ESRR results in a change of diagnosis for 18% of women who are diagnosed as having an abnormal thyroid function test during pregnancy using a local, population-based TPRR. An equal number of women (N=45) classified as euthyroid on the basis of TPRR were found to have an abnormal thyroid function based on ESRR.

In theory, ethnic differences in TSH during pregnancy may be explained by genetic differences in thyroid hormone pathway genes[33-36], since ~65 % of the inter-individual variation in TSH levels has been estimated to be due to genetic factors.[37] Furthermore, ethnicity is a wide concept which is most often socially defined by nationality and culture. Alternatively, environmental factors such as diet or racial disparity of maternal hCG levels may be involved as well.[38-40] Subtle ethnic differences in hCG have been shown in other contexts[38,39], but did not explain ethnic TSH differences in a study by Walker *et al.*[15]

Ideally, each laboratory would calculate both trimester and ethnicity-specific reference ranges for serum TSH. Since ethnic differences within one population from one geographical area already resulted in such a significant misclassification of thyroid disease in our hospital, it is likely that the use of fixed trimester-specific cut-offs (*i.e*. 2.5 mU/L in the first, and 3.0 mU/L in the second and third trimester) throughout the world will result in an even larger number of misclassified patients. It is therefore important to incorporate at least regional trimester-specific reference ranges, if no trimester-specific reference ranges are available in the laboratory.

To date, this is the largest and most detailed study evaluating ethnic differences in thyroid parameters during pregnancy. Moreover, no other study has yet investigated the diagnostic effects of the use of ESRR. A limitation of this study may be the size of some subgroups, especially the size of the Moroccan subgroup (N=308) was relatively small. In addition, we were unable to evaluate disease prevalence per trimester, since most of the samples were obtained in the second trimester. However, ethnic group comparisons are unlikely to be affected as the three largest groups were equally distributed over the first and second trimester. We were unable to fully exclude the effects of thyroglobulin antibodies, however, such antibodies are less common than TPOAbs and in the majority of cases coincide with TPOAb positivity.[41] Finally, even though our data indicate that there are no differences in iodine status amongst the four subgroups, iodine and creatinine data were only available in a random sample of pregnant women.

In conclusion, we have shown that TPOAb status, TSH levels and T4 levels differ significantly according to ethnicity in pregnant women living in an iodine sufficient area. The use of ethnicity-specific

reference ranges instead of a total-population reference range changed the diagnosis for 18% of women who were initially diagnosed as having an abnormal thyroid function test. In order to diagnose and treat pregnant women with (subclinical) thyroid disease correctly, the establishment of reliable reference ranges is of paramount importance. It is likely that ethnic differences similar to the ones shown in this study are present in other populations, but there is currently not enough evidence to incorporate ethnicity specific reference ranges in daily practice. Therefore, further investigations on racial differences in thyroid hormone parameters and their diagnostic and clinical consequences in different regions of the world are warranted.

APPENDIX

SUPPLEMENTAL TABLE 1 *Thyroid parameter differences during pregnancy according to ethnic origin.*

	Total population	Dutch	Moroccan			Turkish	Surinamese			Adjusted *P*-value[a]
Ethnic origin (N)			Arabic (99)	Berber (159)	Unspecified (50)		Creoles (151)	Hindustani (190)	Other (109)	
Adjusted mean TSH (mU/L)	1.40	1.50	1.26*	1.34	1.18	1.48	1.23*	1.45	1.28*	<0.01
Reference range	0.06 – 4.08	0.11 – 4.18	0.003 – 3.91	0.18 – 3.69	0.001 – 3.84	0.04 – 4.26	0.01 – 4.10	0.0002 – 4.01	0.001 – 3.42	
Adjusted mean FT4 (pmol/L)	15.1	15.1	15.3	14.7	15.0	15.1	14.8	15.7*	15.3	0.01
Reference range	10.6 – 21.9	10.8 – 21.8	10.8 – 23.8	8.7 – 20.6	10.4 – 30.0	9.8 – 22.3	9.9 – 25.9	10.7 – 26.2	10.1 – 22.8	
Adjusted mean T4 (nmol/L)	150	142	154*	148	148	156*	14	160*	150	<0.01
Reference range	96 – 219	96 – 204	106 – 232	90 - 221	89 - 267	104 – 238	92 - 243	103 – 254	87 – 222	
TPOAb positivity N (%)	224 (6.1)	151 (5.8)	4 (4.5)	8 (5.4)	2 (4.3)	36 (9.3)*	5 (3.6)	14 (8.0)	4 (4.0)	0.14
TSH >2.5 mU/L 1st trimester N (%)	122 (14.8)	96 (15.5)	0 (0)	1 (6.2)	0 (0)	8 (10.8)	5 (14.3)	10 (27.8)	2 (8.3)	0.42
TPOAb positive women excluded	88 (12.0)	70 (12.6)	0 (0)	1 (7.1)	0 (0)	6 (9.5)	4 (12.9)	5 (19.4)	1 (5.3)	0.88
TSH >3.0 mU/L 2nd trimester N (%)	278 (9.2)	199 (9.5)	4 (4.9)	7 (5.1)	2 (4.8)	46 (13.6)*	4 (3.7)*	12 (8.0)	4 (4.8)	<0.01
TPOAb positive women excluded	192 (7.1)	138 (7.3)	2 (2.8)	5 (4.0)	2 (5.4)	32 (11.0)*	3 (2.9)	8 (6.1)	2 (2.6)	0.03

** Statistically significant (P<0.05) compared to Dutch group. [a] Adjusted for gestational age at sampling, maternal age, SES, smoking, parity and BMI.*
Mean values were calculated as the mean for the 2.5th - 97.5th percentiles of TSH, TT4 or FT4 after exclusion of TPOAb positive women and after correction for gestational age. Adjusted P-values were additionally corrected for maternal age, parity, smoking, socio-economic status and BMI.
Reference ranges were defined as the 2.5th – 97.5th percentiles of respective group after exclusion of twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage or fertility treatment, additionally TPOAb positive women were excluded.

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CHAPTER 3

PLACENTAL ANGIOGENIC FACTORS ARE ASSOCIATED WITH MATERNAL THYROID FUNCTION AND MODIFY HCG-MEDIATED FT4 STIMULATION

Tim I.M. Korevaar, Eric A.P. Steegers, Yolanda B. de Rijke, W. Edward Visser, Vincent W.V. Jaddoe, Theo J. Visser, Marco Medici, Robin P. Peeters

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ABSTRACT

CONTEXT The thyroid has a high vascular density and this vascularity may be influenced by pregnancy-specific angiogenic factors. Proangiogenic placental growth factor (PlGF) and antiangiogenic soluble FMS-like tyrosine kinase-1 (sFlt1; a vascular endothelial growth factor (VEGF) and PlGF antagonist) are important pregnancy-specific angiogenesis regulators. We previously showed that fetal levels of sFlt1 and PlGF are associated with newborn thyroid function. However, the maternal thyroid may also be affected as PlGF and VEGF are secreted into the maternal circulation and cause a concomitant increase of sFlt1 to overcome adverse effects of angiogenesis overstimulation.

DESIGN, SETTING, AND PARTICIPANTS Maternal sFlt1, PlGF, TSH, FT4 or hCG levels were determined during early pregnancy (<18 weeks) in 5517 women from the Generation R study. Analyses were adjusted for relevant covariates and interaction between hCG and angiogenic factors was investigated.

RESULTS Increasing levels of sFlt1 were associated with a decrease in FT4 and T4 (both $P<0.001$), and an increased risk of subclinical hypothyroidism (OR for high levels 2.37 (1.16-4.83), $P=0.02$) and isolated hypothyroxinemia (linear $P=0.02$; OR 3.05 (1.42-6.55), $P=0.004$). Increasing levels of PlGF were associated with a decrease in TSH and FT4 levels (both $P<0.001$), and an increased risk of isolated hypothyroxinemia (linear $P=0.002$; OR 1.77 (1.02-3.06) $P=0.04$). High levels of hCG decreased the difference in FT4 between low and high sFlt1. In women with high PlGF levels, the hCG-mediated increase in FT4 levels was attenuated.

CONCLUSION sFlt1 and PlGF are novel determinants of maternal thyroid (dys)function during early pregnancy and the response of the maternal thyroid function to hCG stimulation. These data provide novel insights into the pregnancy specific thyroid function physiology and suggest that high levels of pro- and anti-angiogenic factors may be a risk factor for adverse pregnancy outcomes via their effects on maternal thyroid function.

INTRODUCTION

Thyroid hormone (TH) plays an important role in the regulation of metabolism and fetal development during pregnancy. Maternal thyroid dysfunction during pregnancy is associated with an increased risk of adverse outcomes including preterm delivery, preeclampsia, low birth weight, and impaired child neurocognitive development.[1,2] During pregnancy many factors determine the maternal thyroid function including iodine levels, placental deiodinase type 3 and thyroid stimulation by increasing levels of placental human chorionic gonadotropin (hCG). However, these mechanisms do not explain all the variability in thyroid function during pregnancy. Another major change in pregnancy-specific physiology is the increase in angiogenic factors produced by the placenta such as Placental Growth Factor (PlGF) and soluble FMS-like tyrosine kinase-1 (sFlt1 or soluble VEGFR-1).[3] The thyroid is a highly vascularized organ yet only little is known on the association of pregnancy-specific changes in angiogenic factors with maternal thyroid function.

Angiogenic PlGF and antiangiogenic sFlt1 are predominantly produced by placental trophoblasts and are crucial for the maintenance of pregnancy and fetal growth.[4] PlGF is a proangiogenic factor which shares a 53% homology with vascular endothelial growth factor (VEGF)[5] whereas sFlt1 is a potent soluble antagonist of VEGF and PlGF signalling. We have previously shown that in cord blood samples from newborns, increasing levels of sFlt1 were associated with an increase in TSH and a decrease in FT4, while increasing levels of PlGF levels were positively associated with FT4 levels.[6] However, both PlGF and (free) VEFG reach the maternal circulation because both of these factors stimulate local placental angiogenesis on the maternal side and may also diffuse into the maternal circulation from the fetal side.[7,8] Subsequently, it has been hypothesized that in order to protect the mother from excessive neovascularization, sFlt1 is secreted into the maternal circulation in a polarized fashion.[9,10]

Evidence suggests that these angiogenic factors may influence the fully developed thyroid gland as well. Animal studies have shown that sFlt1 can reduce thyroid gland vascular density up to ~68% and also cause severe suppression of endothelial fenestration formation and an increase in TSH or decrease in FT4 levels.[11,12] A clinical study by Levine *et al*. showed that an increase of maternal sFlt1was associated with an increase of maternal TSH.[13] Even though PlGF has classically been considered a proangiogenic factor, recent evidence has shown that excess expression of PlGF may exert antiangiogenic effects by inhibiting Braf and ERK activation and via the formation of less potent homodimers or less potent or functionally inactive heterodimers with VEGF.[14-16] For sFlt1 only antiangiogenic effects have been described, yet it remains unknown whether PlGF has a pro or antiangiogenic effect on the maternal thyroid.

During pregnancy, an increased demand for TH necessitates an upregulation of thyroid function which is mediated via increasing levels of hCG which lead to an increase in FT4 and a decrease in TSH levels. Besides a direct effect on the maternal thyroid function via effects on the thyroid gland itself, factors that can interrupt the hCG-mediated stimulation may also influence the maternal thyroid function. We hypothesize that by affecting the vascularity and perfusion of the thyroid gland, sFlt1 and PlGF could have direct effects on the thyroid gland and its function. On top of this, a decrease in vascularity may decrease the surface area between the thyroid gland and the vascular system which could decrease the exposure to hCG and thus also indirectly effect the maternal thyroid function.

Thyroid autoimmunity, represented by thyroperoxidase antibodies (TPOAbs), also is an important determinant of maternal thyroid function during pregnancy as TPOAb positive women have higher TSH levels and slightly lower FT4 levels during pregnancy.[17] Since TPOAb positive women already are at risk for decreased thyroid function, we hypothesized that the effects of angiogenic factors on thyroid function may be stronger as compared to TPOAb negative women.

In order to investigate the effects of gestational angiogenic factors on the maternal thyroid function, we studied the association of maternal sFlt1 and PlGF with TSH, FT4 and thyroid dysfunction in a large prospective cohort study. Furthermore, we investigated if the presence of TPOAbs would affect the associations between these angiogenic factors and maternal thyroid function. Finally, we also investigated if the stimulation of FT4 by increasing levels of hCG would be different in women with low or high levels of sFlt1 and PlGF.

MATERIALS AND METHODS

Design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[18] In 5810 pregnant women, data on early pregnancy TSH, FT4 and sFlt1 or PlGF levels were available. Women with a twin pregnancy (N=128), pre-existing thyroid disease (N=85), thyroid (interfering) medication usage (N=4) or fertility treatment (N=76) were excluded.

Thyroid measurements

Maternal plasma samples were obtained in early pregnancy (median 13.2 weeks; 95% range 9.6-17.6). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L, <5.4% for FT4 at a range of 14.3-25.0 pmol/L. Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when >60 IU/ml (N=239).[17]

Angiogenic factor measurements

sFlt1 and PlGF concentrations were obtained from the EDTA samples drawn at the same time as thyroid measurements and analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics BV, Hoofddorp, The Netherlands). The between-run coefficients of variation for sFlt1 were 2.8% at 5.5 ng/mL and 2.3% at 34.0 ng/mL. The coefficients for PlGF were 4.7% at 24 pg/mL and 3.8% at 113 pg/mL.

Covariates

Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[19] Information on maternal age, smoking status, socio-economic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands.[18,20] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status.[18] Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). Information on fertility treatment, pregnancy outcome, date of birth, birth anthropometrics, and child gender were obtained from community midwives, obstetricians, and hospital registries.

Statistical analysis

We studied the associations of sFlt1 and PlGF with TSH and FT4 by using multiple linear regression models utilizing restricted cubic splines to allow for non-linearity. Effect modification by hCG and TPOAb positivity was investigated by addition of a product interaction term between sFlt1 or PlGF and hCG or TPOAb positivity (yes/no) to the model and we used the visreg package for visualization of significant (*P*<0.05) effect modification. Reference ranges were determined by the 2.5th - 97.5th percentiles after exclusion of TPOAb-positive women as previously described.[17] Maternal overt hyperthyroidism was defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentiles) FT4; overt hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4 and isolated hypothyroxinemia as a low FT4 with a normal TSH. We studied the risk of thyroid dysfunction using multiple logistic regression models utilizing restricted cubic splines to allow for non-linearity.

To satisfy model assumptions, TSH values were logarithmically transformed. Although sFlt1 or PlGF were not associated with TPOAbs, effect modification of TPOAbs was present and therefore TPOAb positive women were excluded from analyses on the association between angiogenic factors and thyroid function (N=356). Additional adjustment of sFlt1 analyses for maternal PlFG and *vice versa* did not change the results. As previously shown, this study population is considered iodine sufficient.[21]

We used multiple imputation for covariates with missing data. Five imputed data sets were created and pooled for analyses. Smoking, socio-economic status and ethnicity were added to the model (missing due to non-response in 13.0%, 7.2% and 4.1%, respectively). Furthermore, we added gestational age at blood sampling, TSH, FT4, TPOAb, hCG, sFlt1 and PlGF levels, parity, maternal age, maternal BMI and fetal gender to the model (randomly missing ≤3.3%). No significant differences in descriptive characteristics were found between the original and imputed datasets. All statistical analyses were performed using R statistical software utilizing the *rms* and *visreg* package or Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

The final population comprised of 5517 women, predominantly nulliparous (56.7%), non-smoking (73,6%) and of Dutch origin (52.6%) from which measurements were available during early pregnancy (median 13.2 weeks; 95% range 9.6-17.6). Further population descriptives are shown in Table 1.

Effects of sFlt1 and PlGF on TSH and FT4

Figure 1 shows the association of sFlt1 and PlGF with TSH and FT4 levels. There was a significant negative linear association of sFlt1 with FT4 levels. sFlt1 levels were not significantly associated with TSH levels. There was a significant negative association of PlGF levels with TSH levels and FT4 levels. All results remained similar when analyses for sFlt1 were adjusted for PlGF or *vice versa*, and there was no interaction between sFlt1 and PlGF. Exclusion of women with preeclampsia, TPOAb positivity or high TSH/FT4 levels also did not change the results.

Effects of sFlt1 and PlGF on TSH and FT4 in TPOAb positive women

Supplemental Figure 1 shows the association of sFlt1 and PlGF with maternal TSH and FT4 levels stratified according to TPOAb status. The effects of sFlt1 on both TSH and FT4 were stronger amongst TPOAb positive women (*P*difference in slopes=0.008 & 0.03). The effects of PlGF on TSH were stronger amongst TPOAb positive women while the effects of PlGF on FT4 were diminished amongst TPOAb positive women (*P*difference in slopes=0.01 & 0.03).

TABLE 1. *Descriptive statistics of 5517 women.*

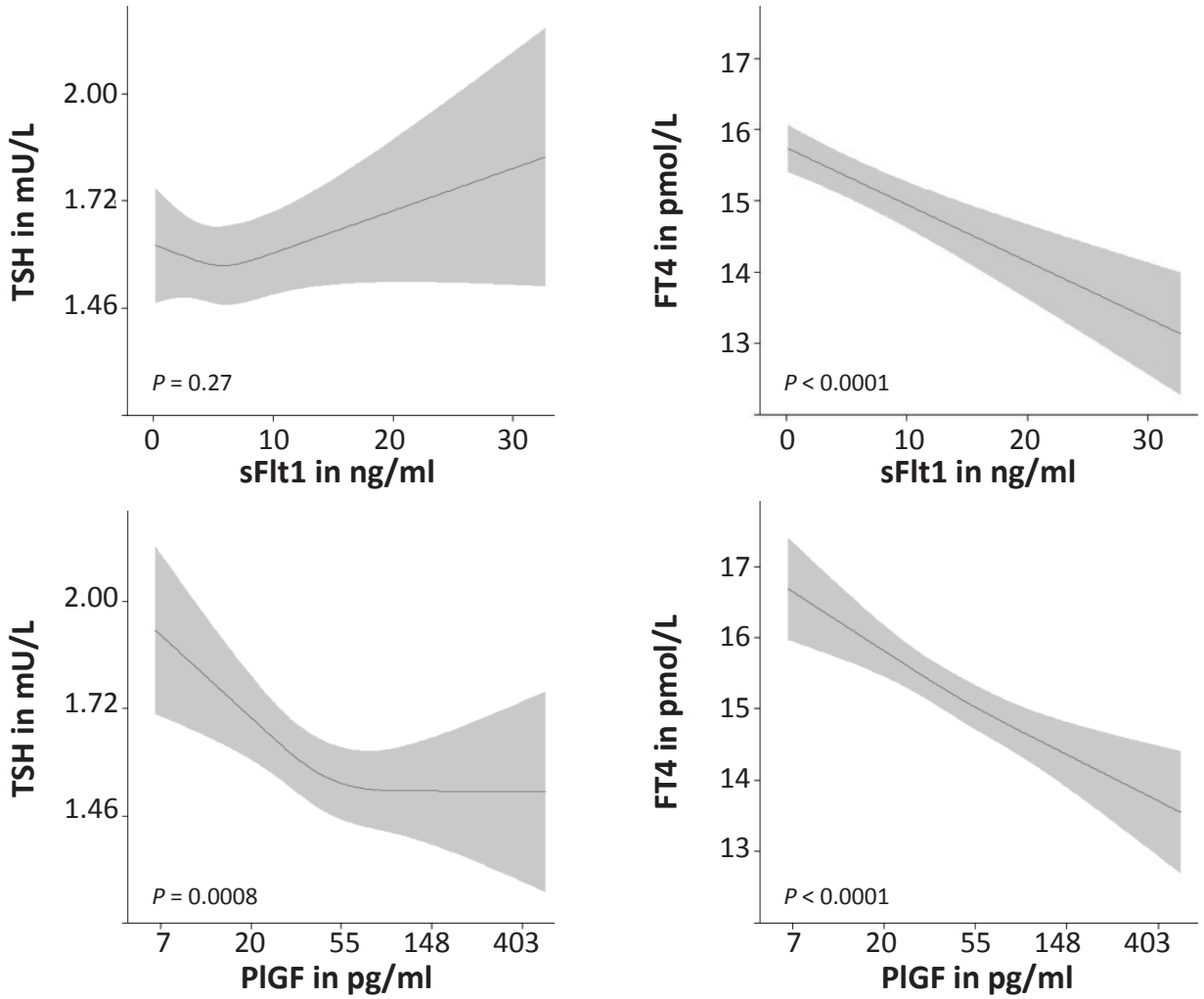
		median	(range)
Maternal age[a]		30.4	(15.3-44.7)
Gestational age at blood sampling[b]		13.2	(4.5-17.9)
TSH	(mU/L)	1.35	(0.00-33.90)
FT4	(pmol/L)	14.8	(6.4-94.6)
hCG	(IU/L)	44,655	(455-187,852)
sFlt1	(ng/mL)	5.04	(0.11-40.1)
PlGF	(pg/mL)	42.8	(6-638)
		N	**(%)**
TPOAb positivity		293	(5.6)
Parity[c,d]			
Nullipara		3143	(60.0)
Primipara		1636	(29.7)
Multipara		739	(13.4)
Smoking[c] (N (%))			
Non-smokers		4056	(73.5)
Stopped smokers		499	(9.0)
Smokers		962	(17.4)
Socio-economic status[c,d]			
Low		581	(10.4)
Middle		2538	(45.5)
High		2398	(44.1)
Ethnicity[c,d]			
Dutch		2850	(51.7)
Moroccan		339	(6.1)
Surinamese		477	(8.6)
Turkish		465	(8.4)
African		314	(5.7)
European		459	(8.3)
Other western		554	(10.0)
Other non-western		58	(1.1)
Body mass index[f]		23.5	(15.2-50.2)
Child gender[c] (% male)		2791	(50.9)

[a] Data shown as median in years
[b] Data shown as median in weeks
[c] Data shown as n (%)
[d] Data shown after imputation of missing data.
[f] Data shown as median in kg/m^2

The association of sFlt1 and PlGF with thyroid disease entities

The associations of sFlt1and PlGF with the risk of subclinical hypothyroidism and isolated hypothyroxinemia is shown in Figure 2. Over the full spectrum, both sFlt1 and PlGF were associated with the risk of isolated hypothyroxinemia but not with the risk of subclinical hypothyroidism. However, as is displayed in Table 2, high levels of sFlt1 were associated with an up to 2.4-fold increased risk of subclinical hypothyroidism and an up to 3.1-fold increased risk of isolated hypothyroxinemia. High levels of PlGF were associated with a 1.8-fold increased risk of isolated hypothyroxinemia. The association of sFlt1 and PlGF with overt hyperthyroidism are shown in Supplemental Figure 2.

FIGURE 1. *The association of maternal sFlt1 and PlGF with TSH and FT4 levels.*



Graphs show the assoiation between maternal levels of sFlt1, PlGF and levels of TSH, FT4 or T4 (black lines) with 95% confidence interval (grey areas). Analyses were performed after exclusion of twin pregnancies, fertility treatment and pre-existing disease or thyroid interfering medication usage and were adjusted for gestational age at blood sampling, hCG levels, maternal age, education level, parity, BMI, ethnicity and fetal gender. Analyses were performed using logistic regression analyses utilizing three restricted cubic splines.

TABLE 2. *High sFlt1 or PlGF levels and the risk of mild thyroid dysfunction during pregnancy.*

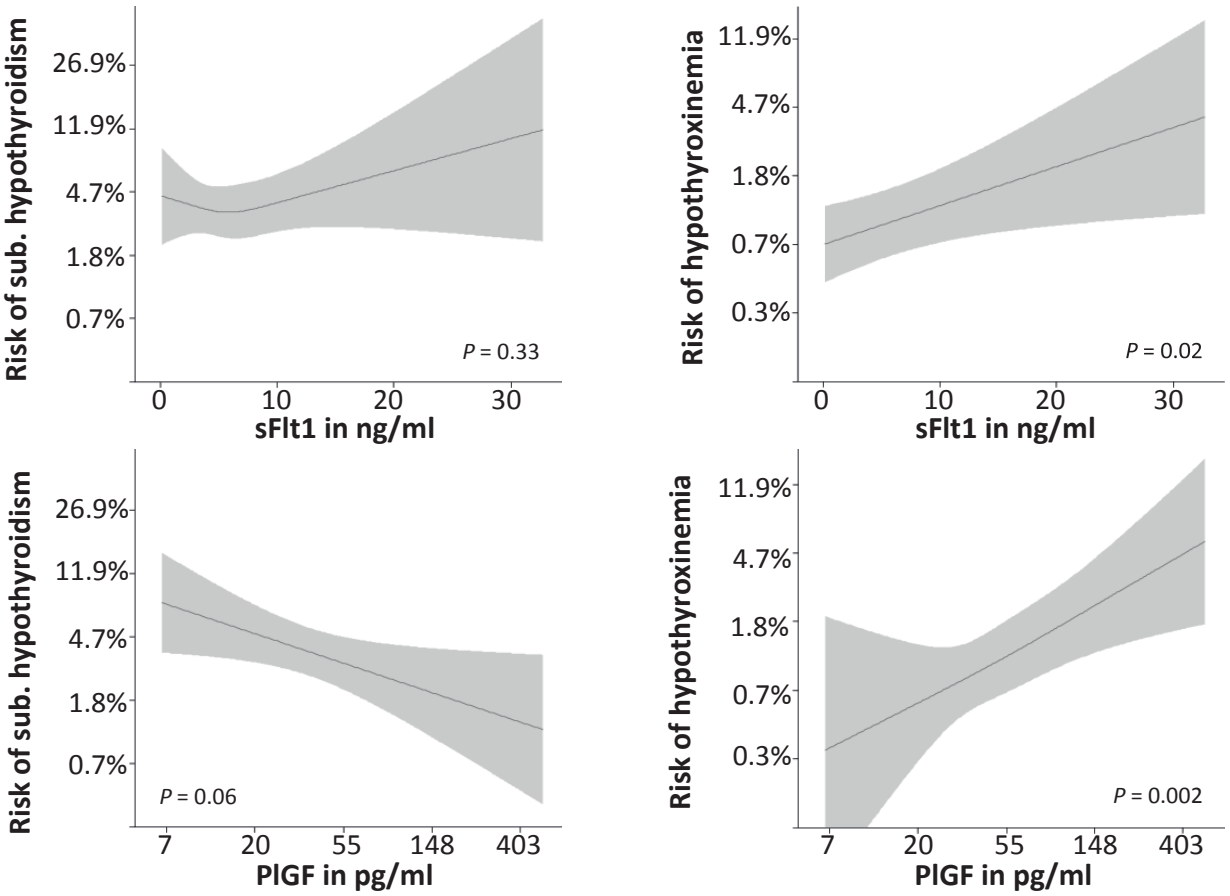
	Subclinical hypothyroidism[a]			Hypothyroxinemia[a]		
	N	(%)	OR (95%CI)	N	(%)	OR (95%CI)
sFlt1 > 95th percentile	14/273	(6.6)	1.85 (1.04-3.29) P=0.04	13/273	(6.6)	3.05 (1.42-6.55) P=0.004
sFlt1 > 97.5th percentile	9/137	(6.6)	2.37 (1.16-4.83) P=0.02	9/137	(6.6)	2.11 (1.12-3.99) P=0.02
PlGF > 95th percentile	6/267	(2.9)	0.75 (0.32-1.79) P=0.52	23/267	(8.8)	1.77 (1.02-3.06) P=0.04
PlGF > 97.5th percentile	4/137	(6.6)	1.01 (0.36-2.85) P=0.99	12/137	(6.6)	1.68 (0.84-3.39) P=0.15

[a] Defined according to the 2.5th and/or 97.5th percentiles for TSH and/or FT4.
Analyses adjusted for gestational age at blood sampling, maternal age, hCG, smoking, SES, ethnicity, parity, BMI and fetal gender.

Effects of sFlt1 and PlGF on hCG stimulation of FT4

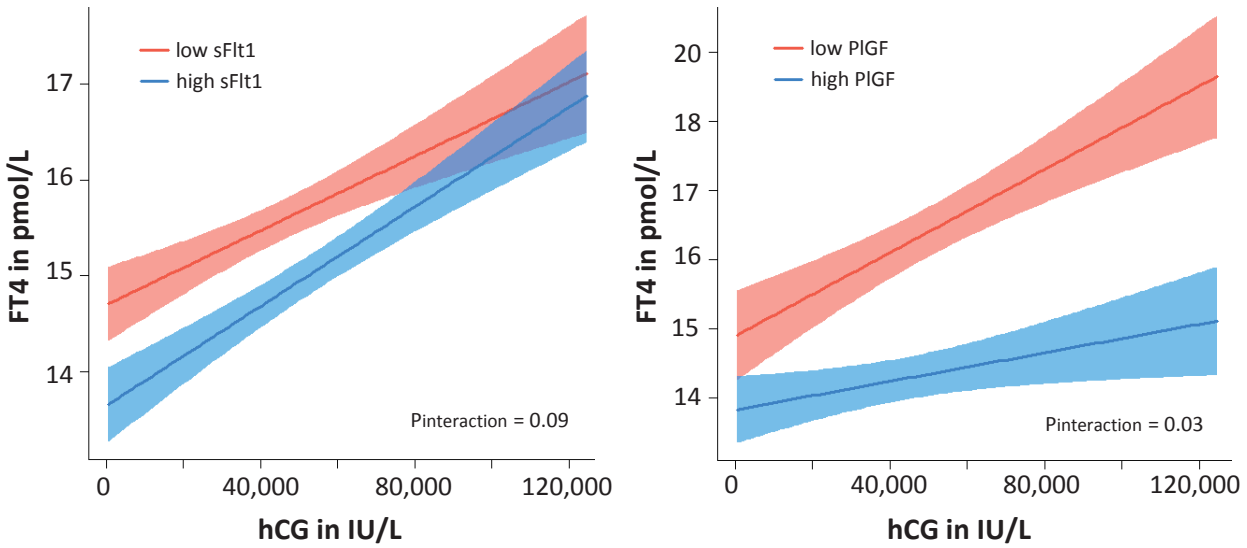
A change in the vascularity and perfusion of the thyroid gland may lead to an impaired response to hCG stimulation. As is shown in Figure 3, the confidence intervals for FT4 levels for women with low or high sFlt1 levels did not overlap when hCG levels were low, this difference was not present when hCG levels were high, and overall this did not reach statistical significance (*P*difference in slopes=0.09). Women with higher levels of PlGF had a lower increase in FT4 levels levels according to hCG stimulation (*P*difference in slopes=0.03).

FIGURE 2. *The association of maternal sFlt1 and PlGF with subclinical hypothyroidism and hypothyroxinemia.*



Graphs show the assoiation between maternal levels of sFlt1, PlGF and proportion of subclinical hypothyroidism or hypothyroxinemia (black lines) with 95% confidence interval (grey areas). Analyses were performed after exclusion of twin pregnancies, fertility treatment and pre-existing disease or thyroid interfering medication usage and were adjusted for gestational age at blood sampling, hCG levels, maternal age, education level, parity, BMI, ethnicity and fetal gender. Analyses were performed using logistic regression analyses utilizing three restricted cubic splines.

FIGURE 3. *The association of maternal sFlt1, PlGF with TSH and FT4 levels.*



Graphs show effect modification by maternal levels of sFlt1 (low and high levels: 1 and 10 ng/ml, respectively) or PlGF (low and high levels: 8 and 148pg/ml, respectively) of the association between hCG and FT4 levels (red/blue lines) with 95% confidence interval (red/blue areas). Analyses were performed after exclusion of twin pregnancies, fertility treatment and pre-existing disease or thyroid interfering medication usage and were adjusted for gestational age at blood sampling, maternal age, education level, parity, BMI, ethnicity and fetal gender. A P-value for interaction of <0.15 was considered for subsequent stratification.

DISCUSSION

In this study we show that maternal angiogenic factors sFlt1 and PlGF are associated with thyroid function during pregnancy. Increasing levels of sFlt1 are associated with a decrease in FT4 levels and an increased risk of isolated hypothyroxinemia. Increasing levels of PlGF are associated with a decrease in TSH and FT4 levels and an increased risk of isolated hypothyroxinemia. In addition, we also show that particularly PlGF may affect the extent by which hCG is able to exert its stimulatory effects on the thyroid. No significant associations were found between sFlt1 or PlGF and subclinical hypothyroidism.

Various risk factors have been identified for mild types of maternal thyroid dysfunction during pregnancy and this knowledge is important in order to further unravel the gestational thyroid pathophysiological mechanisms and develop treatment strategies.[17,20,22-25] Although the thyroid has a high vascular density, data on the effects of pregnancy induced changes in angiogenic factors on thyroid function are sparse. Our results suggest that high levels of circulating angiogenic factors are a risk factor for developing mild thyroid dysfunction. Particularly the clear negative association of sFlt1 with FT4 may help to unravel the pathophysiological mechanism behind isolated hypothyroxinemia, a clinical disease entity that is considered controversial. The potential effects of sFlt1 and PlGF induced thyroid dysfunction on adverse outcomes warrant further investigation.

A previous study by Levine et al. showed that women with high sFlt1 levels had a larger increase in TSH levels between early pregnancy and predelivery but FT4 levels were not available.[13] In newborns, a pronounced negative association between sFlt1 and TSH has been shown while sFlt1 was also negatively associated with newborn FT4 levels.[26] In the current study we observed a non-significant positive trend between sFlt1 and TSH levels, but also that very high levels of sFlt1 levels were associated with an increased risk of subclinical hypothyroidism. This suggests that there is a threshold for sFlt1 to increase

TSH levels. This is also supported by the results of Levine et al. and our previous study in newborns considering that women in the third trimester as well as newborns have higher sFlt1 levels.[13,26] In addition, in our study during early pregnancy the lack of overall effects of sFlt1 on TSH levels but the already present effects on FT4 suggest that also the extent of exposure to sFlt1 plays a role in its effects on the hypothalamic-pituitary-thyroid axis. Although observational studies cannot determine causality, animal experiments strongly suggest that sFlt1 has a direct negative effect on thyroid function by decreasing thyroid vascularization.[11,12]

Data on the association between PlGF and thyroid function are sparse. In newborns, increasing levels of PlGF are associated with an increase in FT4, without effects on TSH.[26] In the current study we found that increasing levels of PlGF are associated with a decrease in both TSH and FT4 levels. The different effects of PlGF on thyroid function in newborns as compared to pregnant women suggest that the stimulation of angiogenesis may be important for proper fetal thyrogenesis while it has different effects on the full grown thyroid gland. Alternatively, the negative association between PlGF and both TSH and FT4 may indicate antiangiogenesis alterations within the pituitary gland, which is also a highly vascularized organ that may be affected by changes in angiogenic factors.[12]

TPOAb positivity is particularly associated with higher TSH but also with slightly lower FT4 levels during pregnancy. In the present study we show that the effects of sFlt1 and PlGF on TSH are stronger amongst TPOAb positive women as compared to TPOAb negative women. For FT4, the effects on sFlt1 were slightly stronger amongst TPOAb positive women. Yet for PlGF, FT4 levels remained at the same level amongst TPOAb positive women, with a very wide confidence interval within this group. There was a large difference between TPOAb negative versus TPOAb positive women in the effects of angiogenic factors on TSH. The difference in strength of the association for TSH versus FT4 fits with the fact that TPOAbs have stronger effects on TSH levels as compared to FT4 levels. Also, this data suggest an amplification effect of the presence of multiple risk factors on changes in thyroid function for the combination of TPOAb positivity and an antiangiogenic biochemistry profile.

Besides the stimulatory effects on the thyroid gland, hCG has also been characterized as a potent stimulator of angiogenesis.[27,28] In the current study we could only demonstrate a small difference in FT4 levels between women with low or high sFlt1 levels when hCG levels are low, but the overall *P*-value for difference between slopes did not reach statistical significance. This suggests that the antiangiogenic effects of sFlt1 on the thyroid gland are rescued by the angiogenic effects of increasing concentrations of hCG. These angiogenic effects of hCG can either be direct angiogenic effects, or might be mediated via stimulation of the TSH receptor. In contrast, the difference in FT4 between women with low or high PlGF levels increased with increasing levels of hCG. Both PlGF and hCG exert their angiogenic effects via stimulation of the cAMP/PKA pathway and it has been shown that the combination of hCG with VEGF has additive angiogenic effects.[27-30] A recent study by Kang *et al*. showed that overexpression of PlGF inhibits angiogenesis in mice during gestation.[14] Taken together, this might suggest that the combined effects of PlGF and hCG can lead to hyperstimulation of angiogenesis, potentially causing antiangiogenic effects which lead to a decrease in thyroid function. Alternatively, a more direct antiangiogenic effect via inhibition of VEGF signaling may also be present.[15,16] However, this is purely speculative and future studies are needed to unravel the physiologic mechanisms behind these associations.

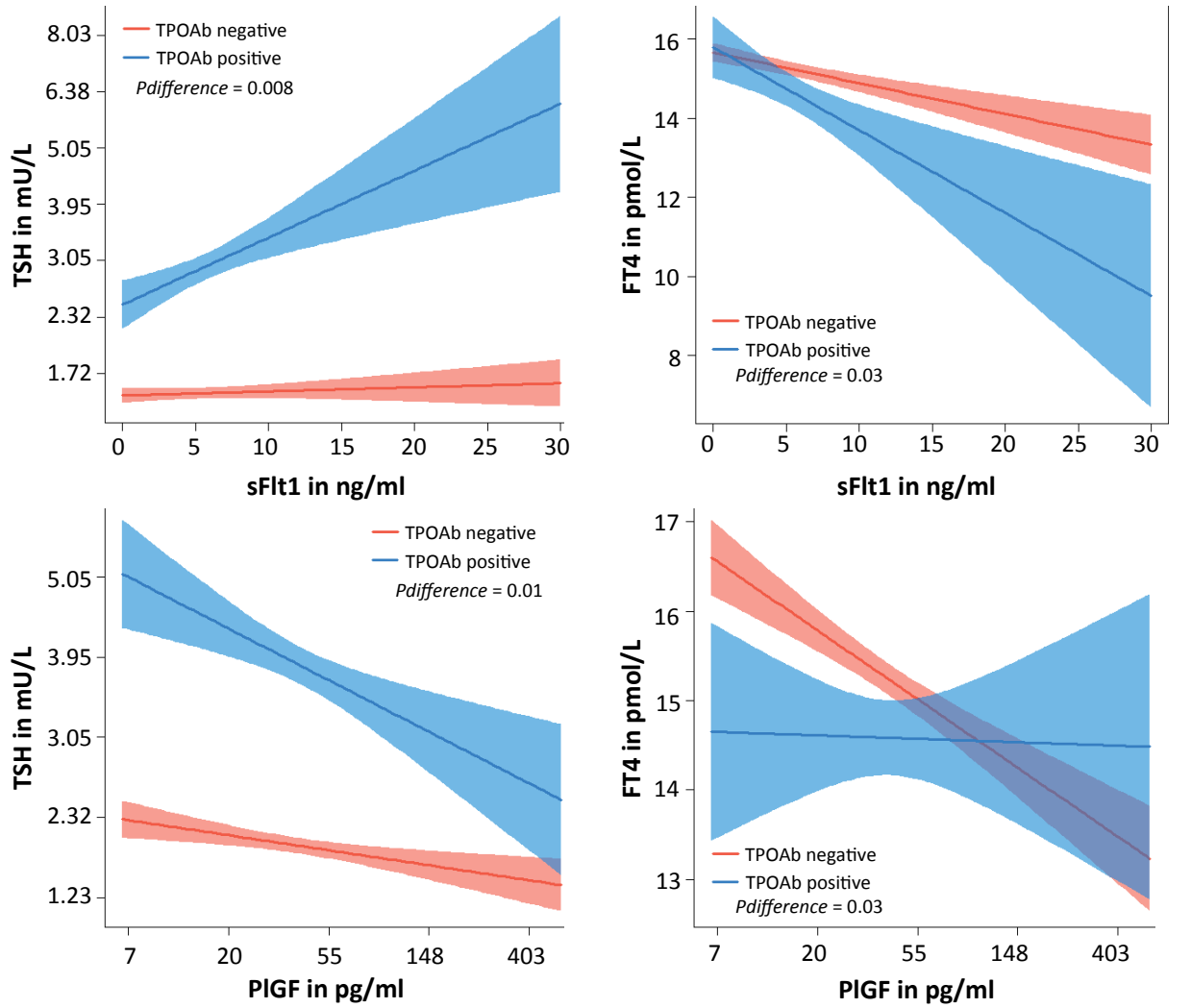
So far, this is the only study that investigated the effects of both maternal sFlt1 and PlGF on TSH and FT4 in pregnant women. We also investigated the effects of sFlt1 and PlGF on the hCG-mediated increase in FT4, which is the main driver of gestational thyroid function changes, and were able to adjust for a wide range of potential confounders. The main limitation of this study is the observational ascertainment of our data, which does not allow to draw conclusions regarding causality. However, recent animal studies, as well as the study by Levine et al. have shown similar effects of increases in

sFlt1 or PlGF.[12-14] Another potential limitation is the fact that all our data were obtained during early pregnancy, which may lead to smaller effects on the thyroid as sFlt1 and PlGF levels are relatively lower during early pregnancy and increase towards the end of pregnancy. On the other hand, this did allow us to investigate the effects of hCG, which reaches highest gestational levels during early pregnancy. The third potential limitation of our study is the lack of power for analyses on overt thyroid disease, even though it is likely that these effects would have the same direction as the effects on subclinical disease.

In conclusion, in this study we demonstrate that early pregnancy levels of maternal sFlt1 and PlGF are associated with thyroid function and the risk of maternal thyroid dysfunction. We also show that the hCG-mediated increase in FT4 is altered by high levels of sFlt1 or PlGF. These data give novel insights into the effects of pregnancy related changes in angiogenic factors on maternal thyroid function. Further studies are needed in order to further clarify the mechanisms behind these associations, for example by performing thyroid ultrasound scans, and investigate to what extent the effects of sFlt1 and PlGF on thyroid function are present outside of pregnancy.

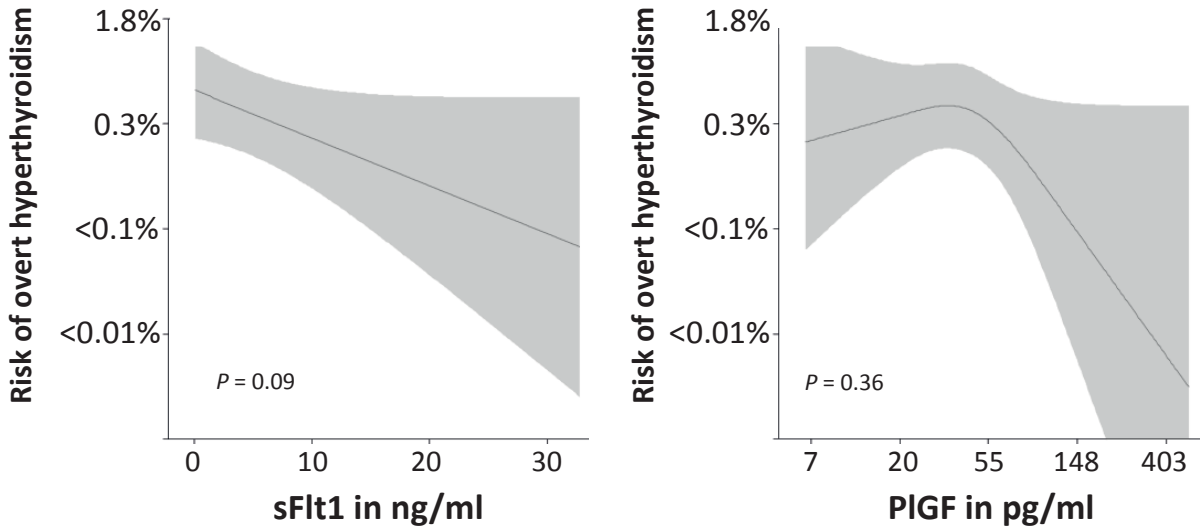
APPENDIX

SUPPLEMENTAL FIGURE 1. *The association of maternal sFlt1, PlGF with TSH and FT4 levels stratified for TPOAb status.*



Graphs show effect modification by TPOAb status (red for negative, blue for positive) on the association of sFlt1 and PlGF with TSH and FT4 with 95% confidence interval (red/blue areas). Analyses were performed after exclusion of twin pregnancies, fertility treatment and pre-existing disease or thyroid interfering medication usage and were adjusted for gestational age at blood sampling, maternal age, hCG, education level, parity, BMI, ethnicity and fetal gender.

SUPPLEMENTAL FIGURE 2. *The association of maternal sFlt1 and PlGF with the risk of overt hyperthyroidism.*



Graphs show the assoiation between maternal levels of sFlt1, PlGF and proportion of overt hyperthyroidism (black lines) with 95% confidence interval (grey areas). Analyses were performed after exclusion of twin pregnancies, fertility treatment and pre-existing disease or thyroid interfering medication usage and were adjusted for gestational age at blood sampling, hCG levels, maternal age, education level, parity, BMI, ethnicity and fetal gender. Analyses were performed using logistic regression analyses utilizing three restricted cubic splines.

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CHAPTER 4

RISK FACTORS AND A CLINICAL PREDICTION MODEL FOR LOW MATERNAL THYROID FUNCTION DURING EARLY PREGNANCY: TWO POPULATION-BASED PROSPECTIVE COHORT STUDIES

Tim I.M. Korevaar, Daan Nieboer, Peter H.L.T. Bisschop, Mariette Goddijn, Marco Medici, Layal Chaker, Yolanda B. de Rijke, Vincent W.V. Jaddoe, Theo J. Visser, Ewout W. Steyerberg, Henning Tiemeier, Tanja G. Vrijkotte, Robin P. Peeters

Clin Endocrinol (Oxf). 2016 Dec;85(6):902-909.

ABSTRACT

BACKGROUND Low maternal thyroid function during early pregnancy is associated with various adverse outcomes including impaired neurocognitive development of the offspring, premature delivery and abnormal birth weight.

AIM To aid doctors in the risk assessment of thyroid dysfunction during pregnancy, we set out to investigate clinical risk factors and derive a prediction model based on easily obtainable clinical variables.

METHODS 9767 women during early pregnancy (≤ 18 week) were selected from two population-based prospective cohorts: the Generation R Study (N=5985) and the ABCD study (N=3782). We aimed to investigate the association of easily obtainable clinical subject characteristics such as maternal age, BMI, smoking status, ethnicity, parity and gestational age at blood sampling with the risk of low free thyroxine (FT4) and elevated thyroid stimulating hormone (TSH), determined according to the 2.5th-97.5th reference range in TPOAb negative women.

RESULTS BMI, non-smoking and ethnicity were risk factors for elevated TSH levels, however, the discriminative ability was poor (range c-statistic of 0.57 to 0.60). Sensitivity analysis showed that addition of TPOAbs to the model yielded a c-statistic of 0.73-0.75. Maternal age, BMI, smoking, parity and gestational age at blood sampling were risk factors for low FT4, which taken together provided adequate discrimination (range c-statistic of 0.72 to 0.76).

CONCLUSIONS Elevated TSH levels depend predominantly on TPOAb levels and prediction of elevated TSH levels is not possible with clinical characteristics only. In contrast, the validated clinical prediction model for FT4 had high discriminative value to assess the likelihood of low FT4 levels.

INTRODUCTION

Adequate thyroid hormone (TH) availability during pregnancy is crucial for the regulation of metabolic demand, energy homeostasis and adequate supply of THs to the developing fetus.[1] Maternal thyroid hormone deficiency occurs in approximately 4.8-18% of all pregnant women depending on the definition used.[2-5] Overt and subclinical hypothyroidism during early pregnancy are associated with pregnancy loss, premature birth, preeclampsia and impaired child neurocognitive development.[2-4,6,7] Recently, hypothyroxinemia (or isolated low FT4 levels) has also been associated with adverse outcomes including premature birth, placental abruption and impaired child neurocognitive development.[8-11]

The fetal thyroid is not fully functional until the 18-20th week of pregnancy. Critical early stages of pregnancy and fetal development therefore predominantly depend on the maternal supply of THs. This specific time window illustrates that it is important to identify women with thyroid dysfunction as early as possible. However, the identification of women at high-risk for gestational thyroid dysfunction in clinical practice is difficult because the majority of women do not present with traditional symptoms. In addition, the status of major known risk factors such as TPO-antibodies and iodine status is usually unknown. This results in under-diagnosis in a large proportion of women with gestational thyroid dysfunction.[12-16]

Because of these reasons, a screening approach is needed to identify women with thyroid dysfunction. However, whether or not all women should be screened for gestational thyroid disease is a matter of controversy and debate. The American Thyroid Association (ATA) and Endocrine Society guidelines advocate aggressive case finding based on risk factors instead of a universal screening approach. Nevertheless, a substantial number of clinicians prefer to perform universal screening over case finding (~50/50 in Europe and ~75/25 in USA and Asia).[17-21] This state of mind is a reflection of studies showing that 30-89% of cases are missed with a case finding approach using risk factors recommended by international guidelines.[12-16] The risk factors that have been used to provide screening estimates in these studies are based on only few, small studies mainly performed in non-pregnant populations.[2,4]

The combination of non-discriminative clinical symptomatology, the lack of evidence-based risk factors, and the ongoing debate regarding screening underlie the need for clinical prediction tools to identify women with thyroid dysfunction during pregnancy. In order to aid physicians during clinical practice, we aimed to develop and validate clinical risk factors and a prediction model for the identification of women with an impaired thyroid function during early pregnancy based only on subject characteristics that are easily obtainable in clinical practice.

METHODS

Design

This study was embedded in two population-based prospective cohorts in the Netherlands, the Generation R Study (Rotterdam) and the Amsterdam Born Children and their Development (ABCD) study.[22,23] Details on data ascertainment are discussed in the supplemental appendix.

Candidate predictors

For the development of this clinical prediction model we selected variables that are readily available or that can be conveniently obtained in clinical practice. This allow for easy implementation into clinical practice. Variables that were considered possible risk factors for elevated TSH and low FT4 (and as a sensitivity analyses also for TPOAb positivity) were selected based on the literature[24-27] and biological

plausibility, and availability of robust ascertainment in both cohorts and included maternal age, BMI, smoking status, parity, ethnicity, gestational age at blood sampling, a medical history (coded as yes/no) of miscarriage or stillborn. Since ethnicity may differ in other populations, we repeated the model with ethnicity recoded as Western/Non-Western in order to allow generalizability to non-Dutch populations.

Outcomes

Reference ranges were determined by population-based calculations according to the 2.5th - 97.5th percentiles after exclusion of women with twin pregnancies, pre-existing thyroid disease or thyroid (interfering) medication usage, fertility treatment and TPOAb positivity, as recommended by international guidelines.[2-4] In Generation R, the intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L (for Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY); total T4 was not assessed.[28] In the ABCD study, the sensitivity of the TSH assay was 0.1 mU/L, and the interassay coefficient of variation was 5.0% while the detection limit of the free T4 assay was 1.9 pmol/L, and the interassay coefficient of variation ranged between 3.1 and 5.0% (access immunoanalyser of Beckman Coultier Inc. (Fullerton, California)). Subsequently, elevated TSH was defined as a TSH level >97.5th percentile (4.04 mU/L in Generation R, 3.09 mU/l in ABCD study). Low FT4 was defined as an FT4 level <2.5th percentile (10.4 pmol/L in Generation R, 7.39 pmol/L in ABCD study). We also investigated a more liberal cut-off value (5th – 95th percentiles). In Generation R, maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/ml. In the ABCD study, TPOAbs were determined by an enzyme-linked immunosorbent assay (ELISA) [ELIZEN TG Ab (E-CK-96), Zentech, Luik, Belgium and considered positive when >80 kU/l.

Statistical analysis

Logistic regression was used to estimate univariable and multivariable odds ratios with 95% confidence intervals (CIs) for each predictor. A full multivariable model was fitted with all candidate predictors with chosen transformation based on linearity assessment with restricted cubic splines. We selected the number of predictors using backward selection based on the Wald statistic of the pooled regression coefficients, with a *P*-value <0.20 as to keep predictors liberally in the model. The regression coefficients in the final model were multiplied with a shrinkage factor which was estimated with bootstrapping.[29] The final model was also presented as a score chart for easy implementation of the model in clinical practice.

The performance of the resulting model was assessed using cross-validation, fitting the model in the Generation R cohort and subsequently validated in the ABCD cohort and vice versa.[30] Prediction models were also internally validated using bootstrapping to calculate optimism corrected estimates of performance. We subsequently assessed calibration and discrimination of the prediction models. Discrimination refers to the ability of a prediction model to distinguish between patients with and without the outcome of interest. Discriminative ability was quantified using the *c*-statistic, which is equivalent to the area under the receiver operating curve for models predicting a binary outcome. For a model with perfect discrimination the *c*-statistic is equal to 1.0 and a *c*-statistic of 0.5 means that the prediction model is equivalent to a coin toss.

Calibration refers to the agreement between predicted probabilities of the prediction model and the observed outcomes. Calibration was assessed graphically using calibration plots and quantified using calibration-in-the-large (calibration-i.t.l.) and the calibration slope. Calibration-i.t.l. measures whether predicted probabilities are on average too high or too low and should ideally be equal to 0.

The calibration slope measures the average predictor strength and should ideally be equal to 1. We assessed the calibration graphically using calibration plots (see Supplementary Appendix). Missing values of the candidate predictors in the Generation R Study and ABCD study data were multiple imputed (five times). The imputation model included all candidate predictor variables, the outcome variable and several relevant variables descriptive of study subjects. All analyses were performed in each of the completed datasets and final results were pooled using Rubin's rules. ll statistical analyses were performed in R 3.1.2, multiple imputation was done using the *mice* package and model fitting was done using the *rms* package.

RESULTS

The final populations comprised 9767 women, N=5985 from the Generation R cohort and N=3782 from the ABCD cohort (Figure S1). Elevated TSH was observed in 217 (3.6%) and 146 (3.9%) women, low FT4 was present in 166 (2.8%) and 108 (2.9%) women and TPOAb positivity was present in 313 (6%) and 227 (6%) women, in Generation R and ABCD respectively. Descriptive statistics of both populations are shown in Table S1, outcomes of the imputation process are show in Table S2 and S3.

TABLE 1. *Risk factors for elevated maternal TSH during pregnancy.*

		Odds ratios for potential risk factors amongst both populations combined		
Predictor	**Category/Measure**	**Univariable**	**Multivariable**	**Prediction model**
Age	*Per year**	1.05 (1.01, 1.09)	1.03 (0.98, 1.07)	
BMI	*Per point*	1.02 (0.99, 1.04)	1.03 (1.01, 1.06)	1.03 (1.00, 1.05)
Smoking	*No*	ref	ref	ref
	Stopped	0.79 (0.56, 1.12)	0.76 (0.53, 1.08)	0.77 (0.54, 1.09)
	Yes	0.70 (0.50, 0.99)	0.71 (0.50, 1.02)	0.70 (0.49, 0.99)
Parity	*0*	ref	ref	
	1	0.87 (0.68, 1.10)	0.82 (0.64, 1.05)	
	2	0.87 (0.59, 1.29)	0.83 (0.55, 1.24)	
	≥3	0.47 (0.21, 1.06)	0.47 (0.20, 1.08)	
Ethnicity	*Dutch*	ref	ref	ref
	Other western	0.87 (0.60, 1.28)	0.88 (0.60, 1.28)	0.88 (0.60, 1.28)
	Moroccan	0.40 (0.22, 0.73)	0.40 (0.22, 0.76)	0.37 (0.20, 0.68)
	Surinamese	0.37 (0.20, 0.66)	0.38 (0.21, 0.69)	0.35 (0.19, 0.63)
	Turkish	0.76 (0.48, 1.19)	0.82 (0.50, 1.33)	0.75 (0.47, 1.19)
	Asian	1.49 (0.92, 2.42)	1.49 (0.91, 2.43)	1.42 (0.87, 2.31)
	Other non-western	0.56 (0.39, 0.81)	0.57 (0.39, 0.83)	0.55 (0.38, 0.79)
Previous miscarrriage or stilborn	*Yes*	1.03 (0.77, 1.37)	1.02 (0.76, 1.37)	
Gestational age	*Per week*	1.02 (0.97, 1.07)	1.04 (0.99, 1.10)	

[a] Percentage of variable data availability in original dataset. For imputed databases see Supplemental Tables.

Risk factors and prediction model for elevated maternal TSH

Higher levels of maternal BMI and Asian ethnicity were associated with a higher risk of elevated maternal TSH whereas smoking and non-Western ethnicity were associated with a lower risk of elevated maternal TSH (Table 1). The combination of relevant risk factors for elevated maternal TSH levels yielded a c-statistic of 0.57-0.60 (Table 2). This model allowed for the calculation of a predictive risk score that can estimate a subject's risk of elevated TSH between 2% and 7% (Table S4). Sensitivity analyses showed that recoding of ethnicity (to Western vs non-Western) did not change the c-statistic while the use of a more liberal TSH cut-off (>95th percentile) did not yield higher c-statistic (data not shown).

TPOAbs play an important role in the pathophysiology of elevated TSH. In order to investigate whether the poor discriminative ability of the prediction model for elevated TSH was due to the strong association of TPOAbs with elevated TSH we added TPOAbs to the prediction model for elevated TSH. After addition of TPOAbs to the model for elevated TSH, this model yielded a c-statistic of 0.73-0.75 (data not shown). A prediction model for TPOAb positivity (Table S5) itself yielded a c-statistic of 0.50-0.57 (data not shown).

TABLE 2. *Discriminative ability of prediction models for elevated TSH, TPOAb positivity and decreased FT4.*

	Populations combined	***Generation R study***	***ABCD study***
Outcome	**C-statistic [a]**	**C-statistic [b]**	**C-statistic [c]**
Elevated TSH	0.60 (0.57*)	0.58	0.58
Decreased FT4	0.76 (0.75*)	0.75	0.72

Odds ratios (95%CI) for potential risk factors for elevated TSH from a multiple logistic regression model.
*Starting from 30 years of age (no differences in risk below 30 years).

Risk factors and prediction model for low maternal FT4

Higher gestational age, maternal age (from 30 years onwards), BMI, parity and smoking were all associated with a higher risk of low maternal FT4 levels (Table 3). A combination of relevant risk factors predicted low FT4 with a c-statistic of 0.72-0.76 (Table 2; addition of ethnicity to the model did not increase model performance). Stepwise addition of covariates to the model show a built up to the final c-statistic as follows: BMI alone (c-statistic 0.69); former model + gestational age (0.747); former model + parity (0.749); former model + smoking (0.75); former model + maternal age (0.76). Sensitivity analysis showed similar results after recoding ethnicity (Western vs non-Western), for a more liberal FT4 cut-off value (<5th percentile), a wider range for gestational age at blood sampling, and after addition of TPOAbs to the model (Table S6).

A clinical scoring model that can be used for the risk assessment of low maternal FT4 is presented in Table 4. This model allowed for the calculation of a predictive risk score that can estimate a subject's risk of low FT4 that will vary between <0.5% and 27% (Figure 1). The more detailed model can be accessed through an online calculator (per journal request, will be made available upon acceptance). Regression formulas for all models are shown in the supplementary appendix.

TABLE 3. *Risk factors for decreased maternal FT4 during pregnancy.*

		Odds ratios for potential risk factors amongst both populations combined		
Predictor	**Category/Measure**	**Univariable**	**Multivariable**	**Prediction model**
Age	Per year*	1.08 (1.03, 1.13)	1.06 (1.01, 1.11)	1.06 (1.01, 1.10)
BMI**	25 vs. 20	2.20 (1.89, 2.58)	2.02 (1.70, 2.40)	2.05 (1.73, 2.42)
	30 vs. 20	4.21 (3.17, 5.59)	3.59 (2.63, 4.90)	3.67 (2.70, 4.99)
Smoking	No	ref	ref	ref
	Stopped	1.10 (0.71, 1.71)	1.44 (0.91, 2.27)	1.41 (0.89, 2.22)
	Yes	1.72 (1.21, 2.45)	1.65 (1.15, 2.36)	1.60 (1.12, 2.29)
Parity	0	ref	ref	ref
	1	1.31 (0.96, 1.78)	1.11 (0.80, 1.53)	1.12 (0.82, 1.55)
	2	2.16 (1.44, 3.23)	1.35 (0.88, 2.08)	1.42 (0.92, 2.17)
	≥3	4.92 (3.15, 7.70)	2.02 (1.21, 3.37)	2.21 (1.34, 3.63)
Ethnicity	Dutch	ref	ref	
	Other western	1.39 (0.84, 2.29)	1.34 (0.81, 2.23)	
	Moroccan	2.30 (1.45, 3.65)	1.33 (0.82, 2.18)	
	Surinamese	1.93 (1.19, 3.14)	1.33 (0.80, 2.22)	
	Turkish	1.78 (1.06, 2.98)	1.10 (0.64, 1.90)	
	Asian	1.48 (0.68, 3.21)	1.33 (0.60, 2.93)	
	Other non-western	2.14 (1.48, 3.08)	1.65 (1.13, 2.42)	
Previous miscarriage or stillborn	Yes	1.28 (0.88, 1.86)	1.14 (0.79, 1.66)	
Gestational age	Per week	1.41 (1.32, 1.50)	1.35 (1.26, 1.45)	1.37 (1.28, 1.46)

C-statistics for prediction models of increased TSH and decreased FT4.
[*] Optimism corrected value in brackets
[a] Apparent model performance
[b] Developed in Generation R, validated in ABCD
[c] Developed in ABCD, validated in Generation R

TABLE 4. *Clinical prediction score for decreased maternal FT4 levels during pregnancy.*

Age		BMI		Week of pregnancy		Parity		Smoking	
≤30	0	≤20	0	≤10	0	0	0	None	0
31-33	1	21-25	3	11-14	10	1	1	Stopped	3
34-35	2	26-29	9	15-18	20	2	3	Yes	5
36-37	3	≥30	15			≥3	8		
38-39	4								
≥40	5								

Total score	Risk of decreased FT4 (%)	N(%) per group	
Whole population	**2.8**	**9415 (100)**	
≤10	<0.5	818	(8.7)
11-17	0.5 - 1	2173	(23.1)
18-24	1 - 2	2926	(31.1)
25-31	2 - 4	2034	(21.6)
32-37	4 - 7	915	(9.7)
38-45	7 - 15	458	(4.9)
≥46	>15	91	(1.0)

FIGURE 1. *The risk of low FT4 according to the risk score chart*

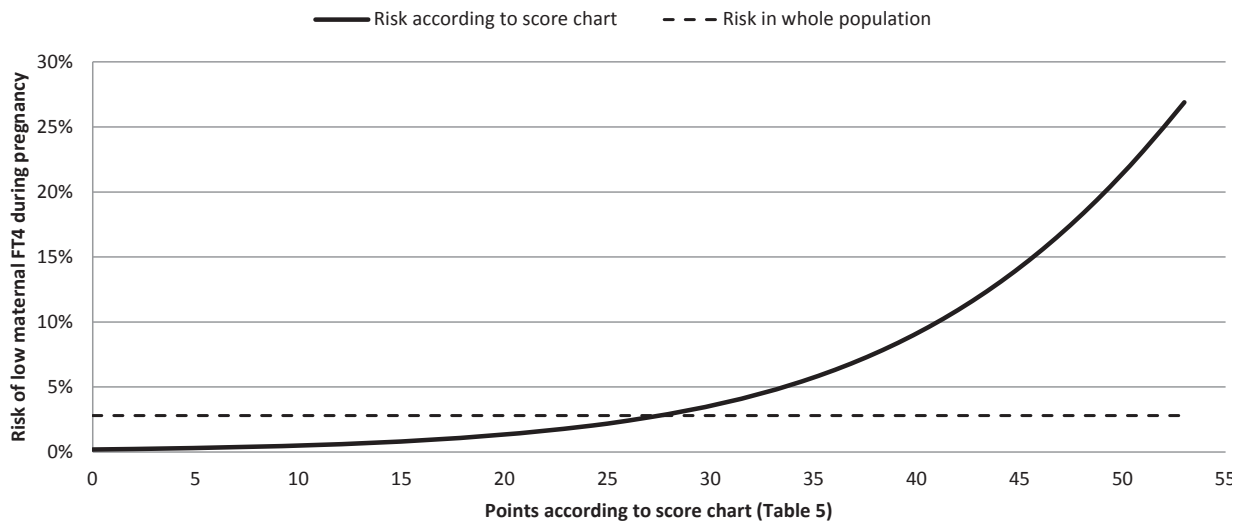


Figure shows the association of the outcome of the risk score with the prevalence of low FT4. The dotted horizontal black line depicts the baseline risk in the whole population.

DISCUSSION

This study reports several easily obtainable clinical risk factors, and the development of the first clinical prediction models for low maternal thyroid function during early pregnancy. The prediction model for low FT4 levels demonstrated a good overall discriminative ability and external generalizability and we provide clinical tools (i.e. a score chart and online calculator) that can be used to assess the risk of low maternal FT4 in clinical practice. We identified several risk factors for elevated TSH levels, however, even a combination of these risk factors lacked proper overall discriminative ability and we show that this is most likely due to the strong association of TPOAbs with elevated TSH.

The American Thyroid Association and Endocrine Society guidelines recommend aggressive case-finding screening utilizing risk factors such as a medical history of head/neck radiation, family history of thyroid dysfunction, obesity (BMI >40kg/m^2), age (>30 years) or symptoms of thyroid dysfunction.[2,4] These recommendations are based on twelve studies of which the majority (11 out of 12) were performed in non-pregnant populations and lacked (cross) replication.[2,4] However, the majority of women with gestational thyroid dysfunction do not have classical or distinct symptoms. Moreover, current risk factors recommended by international guidelines are poorly associated with the risk of abnormal thyroid function and are only present in a very small number of women.[12-16] Because currently used clinical risk factors do not enable to distinguish the low from high-risk groups, physicians are forced to choose between universal screening or screening of a very selected group with distinct symptomatology. Both approaches are suboptimal with regards to efficiency and the ratio of benefits over harms. This study aimed to optimally study risk factors for a risk assessment based on a high-case finding approach, this should allow for better comparison of screening approaches in the future.

Low FT4 levels are associated with impaired child neurocognitive development, abnormal birth weight and a higher risk of premature birth.[8,31-33] Assessment of the pre-test risk is a prerequisite for evidence –based medical decision making, yet in the majority of cases the risk of thyroid dysfunction can only be assessed by expert opinion. Our prediction model provides a clinical tool for assessing the pre-test risk of low FT4 which, together with clinical expertise, will allow clinicians to make an

informed decision on whether or not to test the patient. The prediction model for low FT4 has a good discriminative ability and is particularly able to identify a large number of women that can be considered as a low-risk group. This suggests that the use of the prediction model in clinical practice can optimize both universal and case finding screening strategies. Currently, the effects of levothyroxine treatment in women with isolated low FT4 are unknown and most international guidelines do not advocate treatment.[2-4] It is important to note that the optimal cut-off for any prediction model is based on the benefits (identification of at risk women and providing treatment) and harms (missing at risk women, overtreatment) for each specific cut-off. In order to determine such a cut-off, knowledge on the downstream effects of treatment are required, and such information is currently not available in the field. Therefore, our model can currently be used to assess risk, replacing or adjacent to current known risk factors. However, until further data is available on the optimal clinical decision cut-offs for maternal TSH and FT4 during pregnancy and the harms and benefits of treatment, it is not possible to identify single cut-offs within our model that will optimally distinguish women who will benefit from thyroid function testing.

The small number of women with both elevated TSH and low FT4 levels in the two cohorts (0.2% and 0.7%) and the difference in risk factors for elevated TSH and low FT4 suggest that there is a different pathophysiological mechanism behind these abnormal thyroid function test outcomes. TPOAbs are considered to be a major risk factor for gestational thyroid dysfunction, yet in this study TPOAbs were only associated with a higher risk of elevated TSH. The poor discriminative ability of the prediction model for elevated TSH positivity is explained by the strong association of TPOAbs with elevated TSH as is supported by the results of our sensitivity analysis showing that addition of TPOAbs to the prediction model for elevated TSH substantially improved the discriminative ability. To further substantiate this, we performed a further sensitivity analysis which showed that a prediction model for TPOAb positivity, utilizing the same risk factors as the prediction model for elevated TSH, yielded a poor discriminative ability. Notably, the prediction model for high TSH that incorporates TPOAbs would defeat the purpose of our study and was therefore only devised to investigate the reason for the poor predictive ability of the model. The overlap between TPOAb positivity and elevated TSH also plays an important role in association studies on adverse clinical outcomes as it has been shown that the effect estimates for elevated TSH are much higher with increasing TPOAb levels.[31,34,35] Interestingly, addition of TPOAbs to the prediction model for low FT4 did not improve prediction, although it is likely that TPOAbs are a risk factor for low FT4.

The association of a potential risk factor with TPOAb positivity may also underlie the associations of risk factors with high TSH or low FT4. For example, higher parity was associated with a lower risk of TPOAb positivity while it showed a lower risk estimate for high TSH and a higher risk estimate for low FT4. Interestingly, smoking was associated with a lower risk of TPOAb positivity which fits with data showing that smoking protects against the development of TPOAbs and Hashimoto's disease.[36] In line with this results, smoking was associated with a lower risk of high TSH, yet it was associated with a higher risk low FT4. This discrepancy is likely to represent two different pathways via which smoking can affect thyroid function. Apart from the differences in TPOAb positivity, smoking has also been shown to decrease hCG levels and via this pathway it may affect FT4 levels.[37]

This study was designed for easy implementation into clinical practice, and included a large number of participants with detailed information on clinical characteristics. We used state of the art prediction modelling techniques that aim to accurately predict future patients rather than predictions that are merely correct for patients of the development dataset. The main limitation of this study is that some of the variables were self-reported and derived in a cross-sectional manner. However, this mimics clinical practice where the patient interview is a crucial part of medical decision making.

Another potential limitation is that data on certain variables were derived differently in the Generation R study as compared to the ABCD study. Therefore the external replication analyses may have been suboptimal. The main differences between data derived in the Generation R Study compared to the ABCD study were; measured versus self-reported BMI, measured versus registry derived gestational age at blood sampling, self-reported versus registry derived parity, respectively, and differences in assays used to measure TSH, FT4 and TPOAb levels. In general, differences in assay usage affect the generalizability of virtually all research outcomes in this field, however in this paper we show that our results are replicable for two different assays when population-based reference ranges are used. This is despite differences in absolute levels of TSH and FT4 cut-offs, which are most likely due to the different assays used, but could also be due to population differences in subject characteristics.[38,39] Moreover, it is important to note that although absolute values are likely to differ between populations, relative values are highly correlated all clinically used assays which is illustrated by excellent replication of the prediction model for low FT4.[38]

Although we found similar results after re-categorizing ethnicity groups, further studies are needed to verify if the results of this study are also applicable to other populations. In addition, it should be noted that self-reported variables such as smoking and BMI (ABCD only) may lead to measurement error that may introduce bias in this study. However, self-reported variables mimic clinical practice which allows for better generalizability.

In conclusion, the prediction models presented in this study shows that easily obtainable clinical characteristics are useful for estimating the pre-test probability of low maternal FT4 levels during pregnancy. This model may aid doctors in identification of women at risk for hypothyroxinemia and overt hypothyroidism.

SUPPLEMENTAL APPENDIX - METHODS

Generation R Study

Data on early pregnancy TSH, FT4 or TPOAb levels were available for 6278 women. Women with twin pregnancies (N=128), pre-existing thyroid disease or thyroid (interfering) medication usage (N=89) or fertility treatment (N=76) were excluded.

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks; range 4.5-17.9). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/ml.

Determinants and covariates

Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[1] Information on maternal age, smoking status, maternal education level, obstetrical history and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands and grouped according to major ethnic groups in the Netherlands and/or similarity in thyroid function tests.[2,3] Maternal smoking status was classified as no smoking, smoking until known

pregnancy (stopped), and continued smoking during pregnancy. Education level was defined according to finished education as none/primary, secondary or higher. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). Information on fertility treatment and gender of the child were obtained from community midwives, obstetricians, and hospital registries. Medical and obstetrical history were assessed by questionnaires and answers were crosschecked by certified medical doctors. As we determined previously, this study population is iodine sufficient.[4]

ABCD study

Data on early pregnancy TSH, FT4 or TPOAb levels were available for 3974 women. Women with twin pregnancies (N=50), pre-existing thyroid disease or thyroid (interfering) medication usage (N=32) or fertility treatment (N=110) were excluded.

Thyroid measurements

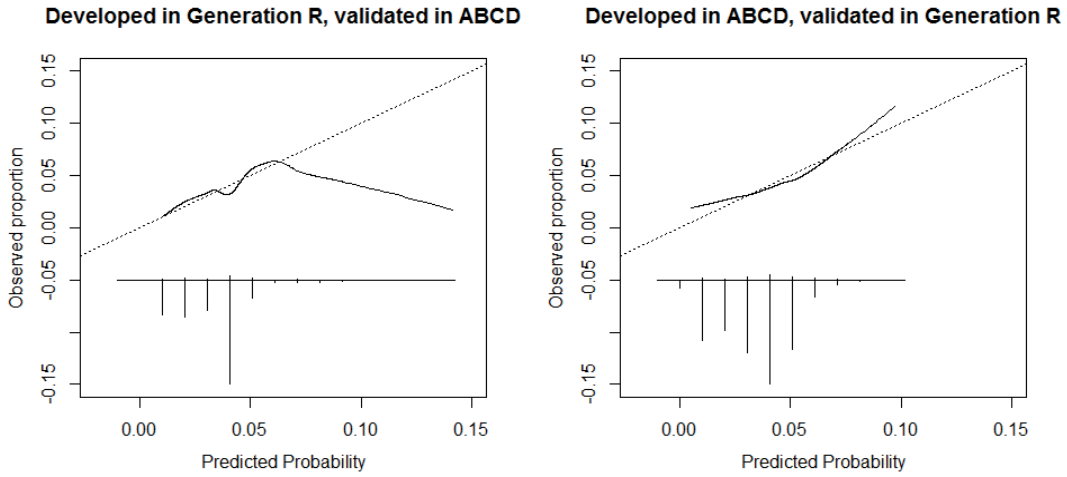
Maternal serum samples selected for replication were obtained during early pregnancy (12.7 weeks; range 4.1-18). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using an access immunoanalyser (Beckman Coultier, Inc.). The intra- and interassay coefficients of variation were <5.0% for TSH and 3.1-5.0% for FT4. TPOAbs were measured using an enzyme-linked immunosorbent assay (ELISA) [ELIZEN TG Ab (E-CK-96), Zentech, Luik, Belgium] and considered positive when >80 IU/ml.

Determinants and covariates

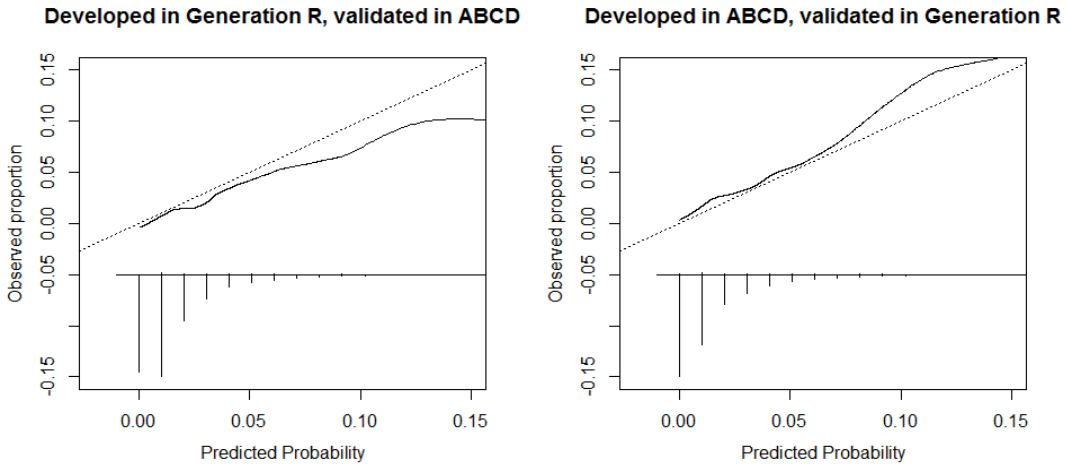
Data on gestational age at blood sampling, parity, child gender, obstetrical history and twin pregnancies were derived from the Youth Healthcare Registration. Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating (~90%) or according to self-reported last menstrual period. Information on maternal age, weight, length (used to calculate BMI), smoking status, years of maternal education, ethnicity, pre-existing thyroid disease, fertility treatment and thyroid interfering medication usage were obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands and grouped according to major ethnic groups in the Netherlands and/or similarity in thyroid function tests.[2,3] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. Education level was defined according to total number of education years after primary school and divided into low (≤4), middle (5-10) or high (>10).

Calibration plots

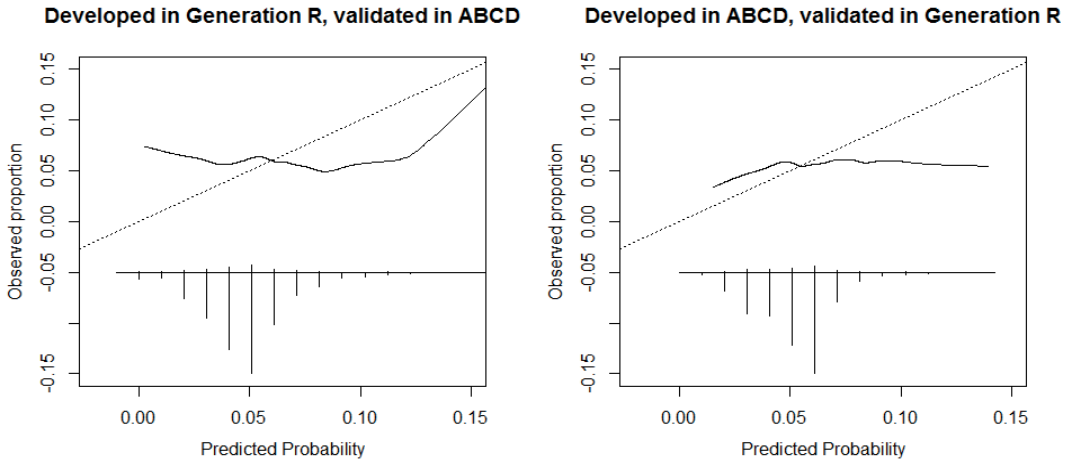
Elevated maternal TSH



Decreased maternal FT4



Maternal TPOAb positivity



Regression formulas

Probability = 1/(1+exp(-lp))

Low FT4

lp = -19.70 + 0.05 * max(age, 30) + 2.13 * log2(bmi) + 0.33 * Smoking = Stopped + 0.45 * Smoking = Yes + 0.11 * Parity = 1 + 0.33 * Parity = 2 + 0.76 * Parity>=3 + 0.30 * Gestage

Elevated TSH

lp = -3.53 + 0.02 * BMI - 0.21 * Smoking=Stopped - 0.29 * Smoking = Yes - 0.10 * Ethnicity = Other Western - 0.8 * Ethnicity = Moroccan - 0.84 * Ethnicity = Surinamese - 0.23 * Ethnicity = Turkish + 0.28 * Ethnicity = Asian -0.48 * Ethnicity = Other non-western

TPOAb positivity

lp = -2.67 + 0.02 * min(age, 30) - 0.09 * Smoking = Stopped - 0.24 * Smoking = Yes - 0.64 * Parity = 1 - 0.12 * Parity = 2 - 0.57 * Parity>=3 - 0.01 * Ethnicity = Other western + 0.05 * Ethnicity = Moroccan + 0.05 * Ethnicity = Surinamese + 0.44 * Ethnicity = Turkish + 0.30 * Ethnicity = Asian - 0.19 * Ethnicity = Other non-western - 0.04 * Gestage

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SUPPLEMENTAL TABLE 1. *Descriptive characteristics of the derivation population and replication population.*

		Generation R study			*ABCD study*		
Variable	**Measure/Category**	**Total N = 5985**		**%N[a]**	**Total N = 3782**		**%N[a]**
Maternal age	Median (IQR)	30.3	(26.2-33.3)	100	32.0	(28.0-34.0)	100
BMI	Median (IQR)	23.5	(21.5-26.4)	99	23.1	(21.3-25.4)	79
Smoking	No	3831	(73%)	87	2786	(74%)	100
	Stopped	481	(9%)		639	(17%)	
	Yes	909	(17%)		353	(9%)	
Parity	0	3407	(57%)	99	2188	(58%)	100
	1	1768	(30%)		1188	(31%)	
	2	552	(9%)		306	(8%)	
	≥3	206	(3%)		100	(3%)	
Ethnicity	Dutch	3023	(53%)	96	2175	(58%)	99
	Other western	520	(9%)		307	(8%)	
	Moroccan	342	(6%)		260	(7%)	
	Surinamese	498	(9%)		214	(6%)	
	Turkish	464	(8%)		147	(4%)	
	Asian	147	(3%)		138	(4%)	
	Other non-western	751	(13%)		540	(14%)	
Previous miscarriage or stillborn	Yes	887	(26%)	57	721	(19%)	100
Gestational age at blood sampling	Median (IQR)	13.2	(12.2-14.9)	98	12.9	(11.9-14.4)	100
TSH	Median (IQR)	1.3	(0.8-2.0)	93	1.2	(0.8-1.7)	99
FT4	Median (IQR)	14.8	(13.0-6.6)	94	9.6	(8.8-10.4)	100
TPOAb levels	Median (IQR)	3.2	(0.0-6.9)	93	0.0	(0.0-0.0)	100
Elevated TSH	Yes	217	(4%)	93	146	(4%)	99
Decreased FT4	Yes	166	(3%)	94	108	(3%)	100
TPOAb positive	Yes	313	(6%)	93	227	(6%)	100

[a] Percentage of variable data availability in original dataset. For imputed databases see Supplemental Tables.

SUPPLEMENTAL TABLE 2. *Descriptive characteristics per imputation in the Generation R study.*

Variable	Measure/ Category	Dataset 1	Dataset 2	Dataset 3	Dataset 4	Dataset 5
Maternal age	Median (IQR)	30.3 (26.2 - 33.3)	30.3 (26.2 - 33.3)	30.3 (26.2 - 33.3)	30.3 (26.2 - 33.3)	30.3 (26.2 - 33.3)
BMI	Median (IQR)	23.5 (21.5 - 26.4)	23.5 (21.5 - 26.4)	23.5 (21.5 - 26.4)	23.5 (21.5 - 26.5)	23.5 (21.5 - 26.4)
Smoking	No	3831 (73%)	4355 (73%)	4373 (73%)	4381 (73%)	4390 (73%)
	Stopped	481 (9%)	566 (9%)	568 (9%)	547 (9%)	550 (9%)
	Yes	909 (17%)	1064 (18%)	1044 (17%)	1057 (18%)	1045 (17%)
Parity	0	3407 (57%)	3433 (57%)	3432 (57%)	3431 (57%)	3435 (57%)
	1	1768 (30%)	1784 (30%)	1788 (30%)	1784 (30%)	1783 (30%)
	2	552 (9%)	557 (9%)	558 (9%)	561 (9%)	558 (9%)
	≥3	206 (3%)	211 (4%)	207 (3%)	209 (3%)	209 (3%)
Ethnicity	Western/ Indonesian	3714 (65%)	3814 (64%)	3809 (64%)	3818 (64%)	3805 (64%)
	Moroccan/ Surinamese	840 (15%)	897 (15%)	901 (15%)	891 (15%)	901 (15%)
	Other non-western	580 (10%)	624 (10%)	624 (10%)	629 (11%)	621 (10%)
	Turkish	464 (8%)	497 (8%)	499 (8%)	491 (8%)	503 (8%)
	Asian	147 (3%)	153 (3%)	152 (3%)	156 (3%)	155 (3%)
Previous miscarriage or stillborn	Yes	919 (27%)	1465 (24%)	1500 (25%)	1459 (24%)	1549 (26%)
TSH	Median (IQR)	1.3 (0.8 - 2.0)	1.3 (0.8 - 2.0)	1.3 (0.8 - 2.0)	1.3 (0.8 - 2.0)	1.4 (0.8 - 2.0)
FT4	Median (IQR)	9.6 (8.8 - 10.4)	9.6 (8.8 - 10.4)	9.6 (8.8 - 10.4)	9.6 (8.8 - 10.4)	9.6 (8.8 - 10.4)
TPOAb levels	Median (IQR)	3.2 (0.0 - 6.9)	3.2 (0.0 - 6.9)	3.2 (0.0 - 6.9)	3.2 (0.0 - 6.9)	3.2 (0.0 - 6.9)
Elevated TSH	Yes	217 (4%)	217 (4%)	217 (4%)	217 (4%)	217 (4%)
Decreased FT4	Yes	108 (3%)	108 (3%)	108 (3%)	108 (3%)	108 (3%)
TPOAb positive	Yes	313 (6%)	313 (6%)	313 (6%)	313 (6%)	313 (6%)

[a] Percentage of variable data availability in original dataset. For imputed databases see Supplemental Tables.

SUPPLEMENTAL TABLE 3. *Descriptive characteristics per imputation in the ABCD study.*

Variable	Measure/ Category	Dataset 1	Dataset 2	Dataset 3	Dataset 4	Dataset 5
Maternal age	Median (IQR)	32.0 (28.0 - 34.0)	32.0 (28.0 - 34.0)	32.0 (28.0 - 34.0)	32.0 (28.0 - 34.0)	32.0 (28.0 - 34.0)
BMI	Median (IQR)	23.1 (21.3 - 25.4)	23.1 (21.3 - 25.6)	23.1 (21.4 - 25.6)	23.1 (21.3 - 25.5)	23.1 (21.3 - 25.5)
Smoking	No	2786 (74%)	2790 (74%)	2788 (74%)	2790 (74%)	2789 (74%)
	Stopped	639 (17%)	639 (17%)	640 (17%)	639 (17%)	639 (17%)
	Yes	353 (9%)	353 (9%)	354 (9%)	353 (9%)	354 (9%)
Parity	0	2188 (58%)	2188 (58%)	2188 (58%)	2188 (58%)	2188 (58%)
	1	1188 (31%)	1188 (31%)	1188 (31%)	1188 (31%)	1188 (31%)
	2	306 (8%)	306 (8%)	306 (8%)	306 (8%)	306 (8%)
	≥3	100 (3%)	100 (3%)	100 (3%)	100 (3%)	100 (3%)
Ethnicity	Western/ Indonesian	2490 (71%)	2707 (72%)	2727 (72%)	2723 (72%)	2710 (72%)
	Moroccan/ Surinamese	474 (14%)	503 (13%)	495 (13%)	496 (13%)	507 (13%)
	Other non-western	239 (7%)	263 (7%)	260 (7%)	257 (7%)	254 (7%)
	Turkish	147 (4%)	158 (4%)	157 (4%)	156 (4%)	163 (4%)
	Asian	138 (4%)	151 (4%)	143 (4%)	150 (4%)	148 (4%)
Previous miscarriage or stillborn	Yes	721 (19%)	721 (19%)	721 (19%)	721 (19%)	721 (19%)
TSH	Median (IQR)	1.2 (0.8 - 1.7)	1.2 (0.8 - 1.7)	1.2 (0.8 - 1.7)	1.2 (0.8 - 1.7)	1.2 (0.8 - 1.7)
FT4	Median (IQR)	14.8 (13.0 - 16.6)	14.8 (13.0 - 16.7)	14.8 (13.0 - 16.7)	14.8 (13.0 - 16.6)	14.8 (13.0 - 16.7)
TPOAb levels	Median (IQR)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)
Elevated TSH	Yes	146 (4%)	146 (4%)	146 (4%)	146 (4%)	146 (4%)
Decreased FT4	Yes	166 (3%)	166 (3%)	166 (3%)	166 (3%)	166 (3%)
TPOAb positive	Yes	227 (6%)	227 (6%)	227 (6%)	227 (6%)	227 (6%)

[a] Percentage of variable data availability in original dataset. For imputed databases see Supplemental Tables.

SUPPLEMENTAL TABLE 4. *Clinical prediction score for elevated maternal TSH levels during pregnancy.*

BMI		Smoking		Ethnicity		Total score	Risk of elevated TSH
≤20	0	No	4	Dutch	8	≤5	2%
20-25	1	Stopped	1	Other western	7	5-10	3%
25-30	2	Yes	0	Moroccan	0	11-15	5%
30-35	4			Surinamese	0	≥16	7%
≥35	5			Turkish	6		
				Asian	10		
				Other non-western	5		

This table shows the clinical prediction score for high TSH (left) and the prevalence of high TSH according to different cut-offs (right).

SUPPLEMENTAL TABLE 5. *Risk factors for maternal TPOAb positivity during pregnancy.*

		Odds ratios for potential risk factors amongst both populations combined		
Predictor	**Category/Measure**	**Univariable**	**Multivariable**	**Prediction model**
Age	*Per year**	1.01 (0.99, 1.03)	1.02 (1.00, 1.04)	1.02 (1.00, 1.04)
BMI	*Per point*	1.00 (0.98, 1.02)	1.01 (0.99, 1.03)	
Smoking	*No*	ref	ref	ref
	Stopped	0.90 (0.68, 1.18)	0.88 (0.66, 1.16)	0.88 (0.66, 1.16)
	Yes	0.72 (0.54, 0.96)	0.72 (0.53, 0.97)	0.72 (0.53, 0.97)
Parity	*0*	ref	ref	ref
	1	0.97 (0.80, 1.18)	0.91 (0.75, 1.11)	0.92 (0.75, 1.12)
	2	0.95 (0.69, 1.30)	0.84 (0.60, 1.17)	0.85 (0.61, 1.18)
	>2	0.50 (0.25, 0.98)	0.44 (0.22, 0.88)	0.45 (0.23, 0.90)
Ethnicity	*Dutch*	ref	ref	ref
	Other western	0.97 (0.70, 1.33)	0.99 (0.72, 1.36)	0.99 (0.72, 1.36)
	Moroccan	0.91 (0.62, 1.33)	1.06 (0.71, 1.57)	1.07 (0.72, 1.59)
	Surinamese	0.92 (0.65, 1.31)	1.06 (0.74, 1.53)	1.07 (0.74, 1.54)
	Turkish	1.46 (1.07, 2.00)	1.82 (1.29, 2.56)	1.84 (1.31, 2.59)
	Asian	1.43 (0.92, 2.24)	1.52 (0.97, 2.37)	1.51 (0.97, 2.37)
	Other non-western	0.71 (0.52, 0.95)	0.76 (0.56, 1.03)	0.77 (0.56, 1.04)
Previous miscarrriage or stilborn	*Yes*	0.98 (0.78, 1.22)	0.99 (0.79, 1.25)	
Gestational age	*Per week*	0.95 (0.91, 0.99)	0.95 (0.91, 0.99)	0.95 (0.91, 0.99)

Odds ratios (95%CI) for potential risk factors for elevated TSH from a multiple logistic regression model.
*Up to 30 years of age (no differences in risk over 30 years).

SUPPLEMENTAL TABLE 6. *Sensitivity analyses on discriminative ability of prediction model for decreased FT4.*

	Populations combined	*Generation R study*	*ABCD study*
Decreased FT4	**C-statistic [a]**	**C-statistic [b]**	**C-statistic [c]**
<2.5th percentile	0.75	0.76	0.72
With TPOAbs	0.78	0.77	0.71
Full gestation[d]	n/a	n/a	0.68
<5th percentile	0.72	0.73	0.68

Sensitivity analyses of prediction models for decreased FT4.
[a] Optimism corrected value

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CHAPTER 5

REFERENCE RANGES AND DETERMINANTS OF TOTAL HCG DURING PREGNANCY

Tim I.M. Korevaar, Eric A.P. Steegers, Yolanda B. de Rijke,
Sarah Schalekamp-Timmermans, W. Edward Visser, Albert Hofman,
Vincent W.V. Jaddoe, Henning Tiemeier, Theo J. Visser,
Marco Medici, Robin P. Peeters

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ABSTRACT

BACKGROUND Human chorionic gonadotropin (hCG) is a pregnancy hormone secreted by the placental synctiotrophoblast cell layer that has been linked to fetal growth and various placental, uterine and fetal functions. In order to investigate the effects of hCG on clinical endpoints, knowledge on reference range (RR) methodology and determinants of gestational hCG levels is crucial. Moreover, a better understanding of gestational hCG physiology can improve current screening programs and future clinical management.

METHODS Serum total hCG levels were determined in 8195 women participating in the Generation R study. Gestational age specific RRs using 'ultrasound derived gestational age' (US RRs) were calculated and compared with 'last menstrual period derived gestational age' (LMP RRs) and a model-based RR. We also investigated which pregnancy characteristics were associated with hCG levels.

RESULTS Compared to the US RRs, the LMP RRs were lower, most notably for the median and lower limit levels. No considerable differences were found between RRs calculated in the general population or in uncomplicated pregnancies only. Maternal smoking, BMI, parity, ethnicity, fetal gender, placental weight and hyperemesis gravidarum symptoms were associated with total hCG.

CONCLUSION We provide gestational RRs for total hCG and show that total hCG values and RR cut-offs during pregnancy vary depending on pregnancy dating methodology. This is likely due to the influence of hCG on embryonic growth, suggesting that ultrasound based pregnancy dating might be less reliable in women with high/low hCG levels. Furthermore, we identify different pregnancy characteristics that influence total hCG levels considerably and should therefore be accounted for in clinical studies.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a pregnancy hormone secreted by the placental synctiotrophoblast cell layer. hCG levels have a very typical trajectory during pregnancy. hCG levels increase exponentially during very early pregnancy, after reaching a plateau during the late first trimester hCG levels steadily decline until a steady state which is seen throughout the second and third trimesters. Classically, hCG is known for maintaining the corpus luteum and its progesterone production, which is essential for embryo implantation.[1-3] Various types of studies have linked hCG to other placental, uterine and fetal functions such as umbilical cord development, suppression of myometrial contractions, the promotion of growth and differentiation of fetal organs but also angiogenesis and regulation of immune tolerance.[4] Although the main clinical utility of hCG levels lies within early pregnancy, these findings underline the importance of hCG throughout gestational physiology and suggest that variations in hCG levels may be associated with adverse clinical outcomes.

Indeed, abnormal levels of hCG have previously been associated with adverse pregnancy outcomes such as fetal loss, preeclampsia, preterm delivery and fetal growth restriction.[5-10] In order to study such clinical associations, it is essential to establish correct gestational age-dependent reference ranges (RRs) which can be difficult because hCG itself has been proposed as a marker of gestational age.[11] hCG has been shown to be and to determine confounding and mediating factors such as differences between different measurement methodologies, pregnancy dating methodologies and differences in population characteristics.[12-15] The latter is especially important because previous studies have demonstrated that certain maternal or fetal characteristics, such as maternal smoking, parity, ethnicity, body-mass index (BMI), placental weight, hyperemesis gravidarum symptoms and fetal gender, that are associated with an increased risk of adverse pregnancy outcomes, are also associated with hCG levels.[16-23]

This study aims to identify determinants of hCG levels during pregnancy that play a role in the complex relationship between hCG and clinical outcomes. We investigated in a large prospective-based cohort study the difference between RRs calculated according to pregnancy dating by ultrasound (US RRs) and RRs determined according to last menstrual period (LMP RRs). In addition, we compared reference range determination by a sensitive model-based approach with the more conventional non-parametric approach and studied if total hCG RRs determined in the general population are different from RRs calculated in uncomplicated pregnancies only. Furthermore, we analyzed which maternal and fetal characteristic are associated with total hCG levels.

MATERIALS AND METHODS

Study population

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[24] In 8195 pregnant women, total serum hCG levels were determined from blood samples drawn from the women at inclusion in the study (median 14.4 weeks; 95% range 10.1-26.2). Women with a late termination of pregnancy (TOP) were excluded from the study population (n=2). For population-based RR, and total hCG determinant analyses, women with twin pregnancies (n=90) or in vitro fertilization treatment (n=38;) were excluded (Supplemental Table 5).

Serum measurements

hCG was analyzed in serum using a solid-phase two-site chemiluminescent immunometric assay, calibrated against WHO 3rd IS 75/537, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The Siemens assay detects serum intact hCG, hyperglycosylated hCG, serum nicked hCG, serum nicked hyperglycosylated hCG, serum asialo hCG, serum hCG free β-subunit and serum nicked hCG β.[25] The inter assay coefficient of variation was 8.0, 6.3 and 5.1% at the concentration of 9.7, 53.1 and 821.5 IU/L, respectively. Although the Immulite 2000 is considered as one of the best assays for total hCG, it should be noted that the reference ranges in this paper are assay specific and do not correspond with hCG values obtained from different assays.[26]

Covariates

Ultrasound examinations were performed using an Aloka® model SSD-1700 (Tokyo, Japan) or the ATL-Philips® Model HDI 5000 (Seattle, WA, USA). Fetal biometry consisting of BPD (outer–outer), HC, TCD, AC and FL was measured during each ultrasound examination. CRL was measured in early pregnancy if feasible and Verburg's equation was used to transform CRL to gestational age.[27] CRL was measured in a true mid-sagittal plane with the genital tubercle and the fetal spine longitudinally in view. The maximum length from cranium to the caudal rump was measured as a straight line. BPD and HC were measured in a transverse section of the head with a central midline echo, interrupted in the anterior third by the cavity of the septum pellucidum with the anterior and posterior horns of the lateral ventricles in view. For BPD the outer–outer diameter was measured perpendicular to the midline and for HC an ellipse was drawn around the outline of the skull. For the TCD measurement the transducer was rotated from the transverse plane for measurement of the BPD towards the cerebellum in the back of the head while keeping the cavity of the septum pellucidum in view. The optimal plane was reached when the peduncles were visualized with a symmetrical shaped cerebellum. The calipers were placed on the outer, lateral edges of the cerebellum. AC was measured in a symmetrical, transverse, round section through the abdomen, with visualization of the vertebrae on a lateral position in alignment with the ribs. The measurement was taken in a plane with the stomach and the bifurcation of the umbilical and hepatic veins using an ellipse around the abdomen. FL was measured with the full length of the bone in view perpendicular to the ultrasound beam. Transvaginal scanning was performed in case of limited visibility by transabdominal scanning in early pregnancy.

Quality checks were carried out frequently to assess the correctness of the ultrasound sections used for biometry measurements and placements of the calipers. Feedback was provided when needed to optimize individual performance. As experience in early pregnancy is limited, intraobserver and interobserver reproducibility of fetal ultrasound measurements from 9 to 14 weeks of gestation was assessed in 21 pregnancies. The intraclass correlation coefficient (ICC) and coefficient of variation (CV) were calculated. The ICC was higher than 0.98 and the corresponding CV lower than 6% for all fetal biometry parameters. Bland and Altman plots to test agreement of measurements for fetal biometry demonstrated normal distributions; the mean difference was around zero and 95% of measurements fell within 2SD of the mean. The 95% limits of agreement for differences in fetal biometry measurements between and among operators in proportions fell within 10% of the mean of the measurements, indicating good reproducibility.[27]

Last menstrual period (LMP) was obtained from the referring letter from the community midwife or hospital. This date was confirmed with the mother at the ultrasound visit and additional information on the regularity and cycle duration was obtained. A subset of 2948 women included during early pregnancy were selected for ascertainment of LMP gestational age, subsequently women with neither a known first day of the last menstrual period nor a regular menstrual cycle of 28 plus or minus 4 days

were excluded (n = 1431). In case of a discrepant result between the LMP obtained from hospital/ midwife letters and self-reported LMP at the research center, the LMP closest to the gestational age based on CRL measurement was used. Information on maternal age, parity, ethnicity, education and smoking status was obtained by questionnaires during pregnancy. Information on fertility treatment, mode of delivery, pregnancy outcome, date of birth, birth anthropometrics, and child gender were obtained from community midwives, obstetricians, and hospital registries.[24]

Statistical analysis

Non-parametric gestational age specific RRs were determined by the 2.5th-97.5th percentiles for each gestational week. In order to compare total hCG values throughout gestation, multiple of median (MoM) values were calculated by dividing each participant's total hCG level with the median value of the total group for that particular gestational week. Model-based reference ranges were created using Generalized Additive Models for Location, Size and Shape (GAMLSS). These specific statistical tools enable flexible, (semi) parametric, RR calculations while accounting for skewness and kurtosis of the data during the modelling process. We used 15 cubic splines for gestational age at blood sampling, 3 cubic splines for sigma variation and a Box Cox *t* family distribution (after sensitivity analyses using Akaike Information Criterion and worm plots) in order to achieve the best fit, while also accounting for the known, typical pregnancy hCG trajectory.[28] Subsequently, gestational age specific Z-scores were derived from the model. In order to compare the model cut-off values to the non-parametric cut-off values (calculated per week), 2.5th, 50th and 97.5th values calculated for the middle of each week were derived from the model.

Because hCG may influence early fetal growth, gestational age that is defined according to fetal growth (US RRs) may differ according to hCG levels. For this reason, we also defined gestational age according to the first day of the LMP in a subgroup of mothers with data available on LMP that had a regular menstrual cycle (28 plus or minus 4 days; n=1526).[29,30]

As hCG levels may differ in complicated pregnancies, RRs were also determined in uncomplicated pregnancies only. For these analyses we selected women with uncomplicated pregnancies by excluding pregnancies with a non-live born child, preterm birth, a small for gestational age newborn, hypertensive disorders or pre-existing hypertension, resulting in a population of n=7015; definitions of complicated pregnancies have previously been described in detail.[31-33]

Since hCG is secreted by trophoblasts, the number of trophoblast cells (approximated by the weight of the placenta) may influence total hCG levels. Therefore, we investigated whether placental weight at birth is associated with total hCG MoM levels. Furthermore, it is speculated that hCG plays a role in hyperemesis gravidarum, and therefore we investigated if specific hyperemesis gravidarum symptoms (reflux/belching, nausea or vomiting) are associated with total hCG MoM levels.

For covariates with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used.[34] Five imputed data sets were created and pooled for analyses. Maternal smoking, education, ethnicity, BMI, parity and child gender were added to the model (missing due to non-response in 12.6%, 9.0%, 5.4% and <2%, respectively).

Furthermore, we added gestational age at time of blood sampling, maternal age, and pregnancy complications as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. Confidence intervals for US RRs were created using bootstrap analyses with 1000 sample draws. The associations between maternal or fetal characteristics and total hCG (MoM) levels were analyzed by ANOVA and linear regression. Univariate analyses were adjusted for gestational age at blood sampling and multivariate analyses were adjusted for gestational age at blood sampling, maternal age, smoking, BMI, education level, maternal ethnicity, parity and child

gender. To achieve normal distribution for statistical testing, total hCG values and MoM values were transformed by the natural logarithm. The above analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Chicago, IL, USA). The associations between pregnancy characteristics and total hCG MoM levels depicted in the figures were assessed by ordinary least squares fitting functions with restricted cubic splines from the RMS library in R statistical package, version 3.03.

RESULTS

Descriptive characteristics of the study population are shown in Supplemental Table 1. Population-based, gestational age specific median and RR values for total hCG are shown in Table 1 and model-based reference centile curves are depicted in Figure 1. Throughout gestation, total hCG levels showed a peak in the 9th and 10th week of gestation, after which a steady decline was observed.

TABLE 1. *Gestational age specific, total population reference ranges for hCG in 8065 women.*

Gestational week	N	Median	Minimum	2.5th	97.5th	Maximum
<9	32	59,973	455	2,305	94,251	142,584
9	50	75,494	22,655	24,310	125,882	129,909
10	106	74,655	16,080	24,370	137,697	163,393
11	255	62,493	10,340	23,669	129,242	187,852
12	790	56,004	8,105	22,846	114,774	164,125
13	1,418	52,367	4,618	23,272	109,990	166,478
14	1,069	47,267	5,925	20,494	105,369	144,054
15	800	37,303	4,834	14,262	82,506	122,037
16	594	29,614	7,512	11,159	80,656	132,084
17	455	24,426	5,637	8,294	69,447	151,558
18	354	20,693	3,822	6,637	50,109	75,993
19	271	17,609	3,895	5,022	52,640	90,628
20	389	17,354	3,128	5,342	43,692	78,841
21	530	15,088	1,542	4,213	42,892	73,485
22	330	16,174	2,559	3,689	44,548	86,541
23	165	12,415	1,957	2,390	43,379	65,192
24	134	13,739	2,511	4,067	45,031	49,392
25	79	14,749	3,354	3,847	53,383	63,166
>25	244	13,852	518	2,228	58,125	74,719

hCG reference range values were calculated according to a population-based approach in the whole study population, after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2).

Reference range comparisons

Pregnancy dating based on ultrasound is determined by fetal size. Considering that hCG is associated with fetal growth, we studied if gestational age specific hCG RRs are different when gestational age is determined by ultrasound (US RRs) or based on the first day of the last menstrual period (LMP RRs). As is shown in Table 2, compared to US RRs, LMP RR levels showed a shift to the left with particularly lower levels for the median and lower limit levels. For RRs determined in women with an uncomplicated pregnancy, only small differences with the population-based approach were seen (Supplemental Table 2).

TABLE 2. *Comparison of reference ranges for total hCG according to gestational age determined by ultrasound or last menstrual period (LMP).*

Gestational week	N Ultrasound	*N LMP*	Median hCG (95% CI)		*LMP*	2.5th percentile (95% CI)		*LMP*	97.5th percentile (95% CI)		*LMP*
11	255	*91*	62,493	(58,665 – 67,327)	*56,780*	23,669	(16,372 – 26,937)	*11,189*	129,242	(111,434 – 160,438)	*132,875*
12	790	*470*	56,004	(54,242 – 58,142)	*52,252*	22,846	(19,793 – 24,392)	*12,193*	114,774	(110,101 – 126,943)	*110,118*
13	1,418	*633*	52,367	(51,237 – 53,893)	*50,596*	23,272	(21,953 – 25,260)	*14,547*	109,990	(103,844 – 116,031)	*105,402*
14	1,069	*323*	47,267	(45,697 – 48,706)	*47,965*	20,494	(17,626 – 21,988)	*12,842*	105,369	(96,283 – 110,567)	*96,874*

Italic numbers = gestational age determined by reliable first day of last menstruation. hCG reference range values were calculated according to a population-based approach in the whole study population, after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2). Gestational age at blood sampling was determined according to ultrasonography measured crown-rump length or first day of last menstrual period, if reliable. 95CIs were determined by bootstrap analyses using 1000 sample draws.

Supplemental Table 3 shows the median, and upper or lower limit cut-off values for total hCG as calculated by the previous non-parametric method compared to the same cut-off values derived from a model-based approach. In general, the model-based RRs were in the low-normal region of the non-parametric RRs 95% confidence interval. However, overall there was not a statistically significant differences between the cut-off values from both methods. Furthermore, the z-scores derived from the model were highly correlated with the commonly used Multiple of Median (MoM) values (Standardized β = 0.919; data not shown).

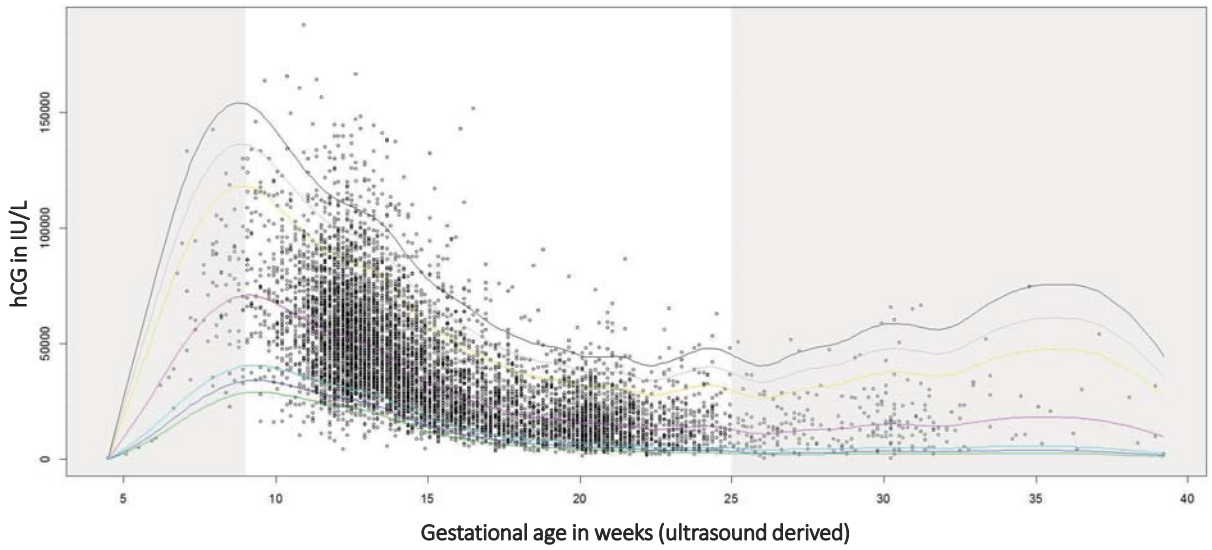
Determinants of hCG

Figure 2 shows the association between maternal or fetal characteristics and total hCG levels adjusted for gestational week by multiple of median (MoM) transformation. Taken together, the determinants depicted explained 6.7% of the variability with maternal smoking, BMI, parity and child gender as the main determinants of total hCG (MoM) levels. Compared to non-smokers, smokers on average had lower total hCG values (-6,299 ± 642 IU/L; *P*<0.001) and the effects of smoking on total hCG levels were dose dependent. The effect of smoking on total hCG levels was modified by gestational age (interaction term 'smoking(yes)'*'gestational age at blood sampling': *P*=0.10; with corresponding β for total hCG MoM level for the first, second (wk 13.1-16.5) and third tertile of gestational age of 0.-143, -0.189 and -0.186, respectively). The total hCG values of women who stopped smoking after a positive pregnancy test were similar to non-smokers. Women within the highest BMI quintile on average had a substantially lower mean total hCG level compared to women within the first quintile (average difference 9,369 ± 729 IU/L, *P*<0.001; Supplemental Table 4) and mean total hCG level differences according to parity and child gender ranged between approximately 2,000-4,000 IU/L. These results remained similar after multivariate correction for potential confounders (Supplemental Table 4). We also investigated the women who were excluded for these analyses and found that IVF treatment and twin pregnancies were associated with higher mean total hCG (MoM) levels (Supplemental Table 5).

As is shown in Figure 3, an increase in placental weight was associated with an increase in total hCG MoM values. In the multivariate model, placental weight remained associated with total hCG levels. Although addition of placental weight to the model did reduce the strength of the associations between BMI, smoking, parity, ethnicity or fetal gender and total hCG (MoM) levels, these associations remained highly significant.

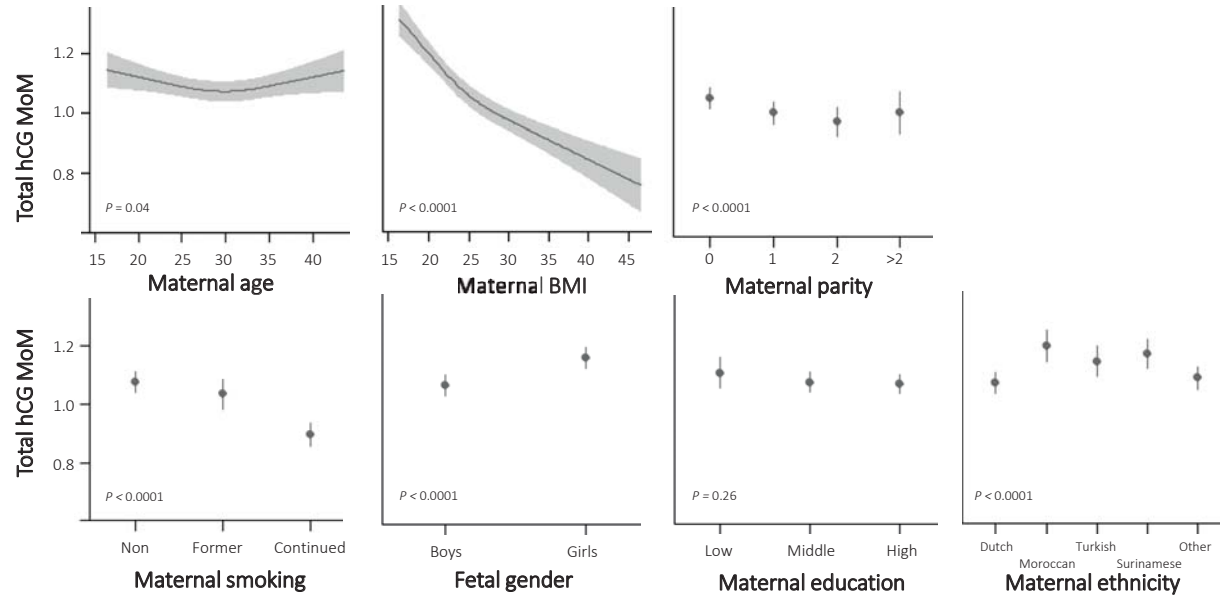
Furthermore, an increasing frequency of self-reported hyperemesis gravidarum symptoms (i.e. reflux/ belching, nausea or vomiting) was associated with an increase in total hCG MoM values (Supplemental Table 6).

FIGURE 1. *Gestational age specific reference ranges for total hCG levels during pregnancy.*



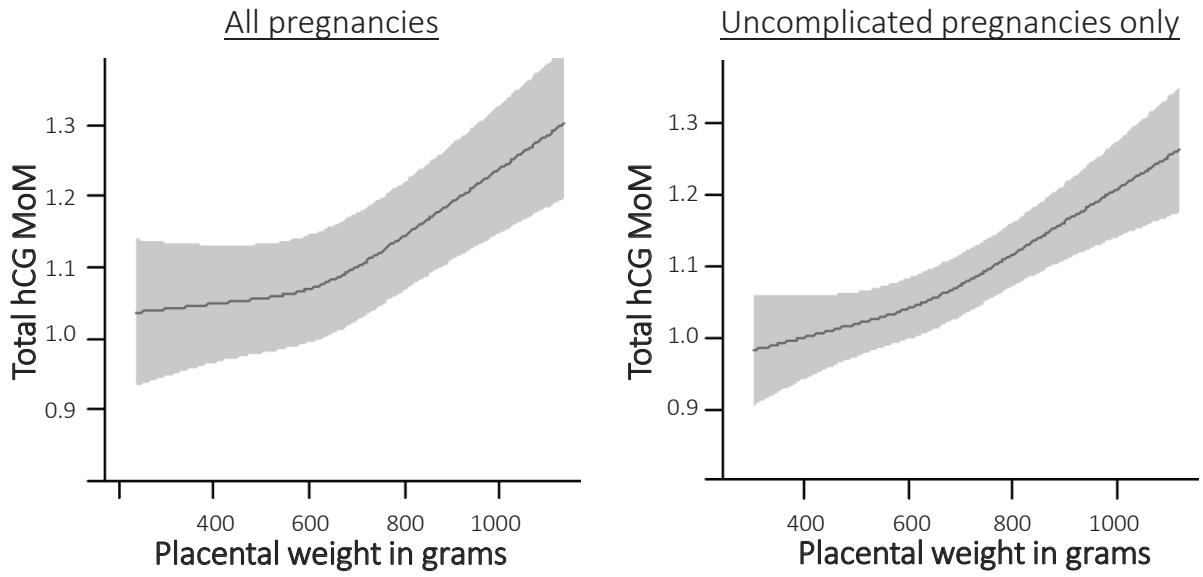
Total hCG reference range values were calculated according to a (semi) parametric 2.5th-97.5th percentiles by GAMLSS modelling in a population-based approach among the entire study population, after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2). Colored lines depict the gestational age specific centiles for total hCG levels. Grey area depict areas with higher uncertainty due to small numbers (N per week <40 before week 9 and after week 24).

FIGURE 2. *The relationship between maternal or fetal characteristics and total hCG MoM levels.*



Plots show the relationship between pregnancy characteristics and total hCG MoM levels for continuous and categorical variables as predicted mean with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2), and were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity and fetal gender.

FIGURE 3. *The relationship between placental weight and total hCG MoM levels.*



Plots show the relationship between placental weight at birth and total hCG MoM levels as predicted mean with 95 percent confidence interval. Analyses were performed after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2; placental weight available in n=5851) and were adjusted for maternal age, smoking, BMI, parity, education level, ethnicity and fetal gender. For uncomplicated pregnancies we selected women's first pregnancy registered in our database and excluded pregnancies with a non-live born child, preterm birth, a small for gestational age newborn, hypertensive disorder or pre-existing hypertension resulting in a population of n=7015 (with placental weight available n=4999).

DISCUSSION

Total hCG values and RR cut-offs during pregnancy vary depending on different methodological as well as individual factors. In the current study we determined a population-based gestational age specific RR for total hCG during pregnancy and we demonstrate that these RRs differ depending on the methodology used to determine gestational age. Furthermore, we show that maternal smoking, BMI, parity, ethnicity, child gender and placental weight are factors associated with total hCG levels and that increasing severity of reflux/belching, nausea and vomiting symptoms was associated with increasing total hCG levels.

We determined RRs for total hCG amongst the whole population and when we compared such RRs with RRs calculated in women with uncomplicated pregnancies we found only small, negligible differences. RRs were also calculated using a model-based approach. Although there was an overall trend for lower estimates as compared to the non-parametric methods, these differences overall did not reach statistical significance. Future analyses should determine whether these differences in cut-off values influence the associations of total hCG with pregnancy complications or whether there are consequences for the identification of women with a clinically relevant increased risk of other adverse outcomes. However, considerable differences were present between the US RRs and the LMP RRs. Overall, US RRs were higher compared to LMP RRs and as such it seems likely that US RRs are affected by the effects of hCG on fetal growth. This fits with observations that hCG levels are negatively associated with fetal growth.[35,36] Moreover, this suggests that pregnancy dating by ultrasound, which is considered the gold standard, might be less reliable in women with relatively high or low levels of hCG.

We show that BMI is one of the most influential determinants of total hCG levels, exhibiting an inverse association. Previous studies have shown a similar association between hCG and BMI, and some

aneuploidy screening programs use BMI corrected values in order to increase testing performance.[18,19,37] The pathophysiology behind these associations is currently unclear. BMI has been positively associated with placental weight and increasing placental weight is associated with increasing hCG levels in this study. This may suggest that higher placental weight in women with high BMI levels may compensate the negative association between BMI and hCG. However, in a subset of women in which placental weight was known (n=5851), the association between BMI and total hCG MoM levels remained similar after adjustment for placental weight (β ± SE per ln(MoM) change; unadjusted: -0.019 ± 0.001 *vs.* adjusted: -0.020 ± 0.001; data not shown) suggesting separate mechanisms in the effects on hCG. The pathways via which this effect occurs remain to be elucidated and a potential role for adipokines or inflammatory markers should be considered.[38-40]

Similar to previous studies, smoking was associated with lower hCG levels in the current study as well. However, we are the first to show that women who stopped smoking when the pregnancy test was positive had similar total hCG levels as non-smokers (Supplemental Table 4). This indicates that discontinuation of smoking at the time of known pregnancy may prevent the reduction in total hCG levels seen amongst continuing smokers and that the effects of smoking on total hCG levels will only become apparent after a particular smoking duration (dose dependency). Indeed, similar to findings by Ball *et al*.[41,42], the strength of the association between total hCG and smoking increased with gestational age. Most likely, this effect is a cumulative smoking effect considering that we also found a strong dose-dependent association between the number of cigarettes smoked and total hCG decrease. For aneuploidy screening, usually utilizing β-hCG levels, neither the total effects of smoking nor the gestational age dependent effects had a considerable impact on the outcome.[16,41,43] Prenatal smoking has consistently been associated with an increased risk of small for gestational age children and low placental weight. It is likely that the effects of prenatal smoking on birth weight of the newborn are at least in part caused by a decrease in hCG levels as it has been shown that prenatal smoking leads to an increase in apoptosis of synctiotrophoblast cell layer.[44] Future studies should investigate to what extent hCG contributes to the changes in fetal growth and birth weight. Moreover, given the unequivocal link between smoking and adverse perinatal outcomes, the strong association between smoking and total hCG levels is a clear demonstration of the confounding potential of pregnancy characteristics in studies investigating the relationship between hCG levels and any clinical outcomes/measurements.

Interestingly, in particular the effects of smoking, but also the effects of other characteristics seemed to be more pronounced in our study compared to other studies.[16-18,20,21,43,45] This may be due to the fact that we determined total hCG levels using an assay which detects the vast majority of hCG variants[25] whereas most other studies report the effects on β-hCG. In turn, this could suggest that BMI, smoking, parity, ethnicity, child gender and placental weight have differential effects on specific types of hCG such as nicked or hyperglycosylated hCG.

To our knowledge, this is the only study which reports RRs for total hCG during pregnancy apart from the manufacturer of the assay that we used, which reported on 593 pregnant women.[46] Furthermore, we are the first to report the associations between detailed maternal and fetal characteristics and total hCG levels during pregnancy. Access to an extensive database allowed us to compare different methods of RR determinations and study the association of various sparsely reported maternal/pregnancy characteristics including placental weight and vomiting symptomatology. We were, however, limited by the fact that LMP and the menstrual cycle, placental weight and vomiting symptoms were only available in a subset of women. Also, the number of women with availability of total hCG measurements varied for each gestational week and therefore reference range determinations were not equally reliable throughout gestation, particularly during very early and the third trimester of pregnancy. Potential differences in formulas used to determine gestational age based on ultrasound data may also underlie some of our results and warrant further research.

In conclusion, we provide data on total hCG reference ranges during pregnancy from a large prospective population-based cohort and identified that these may considerably differ according to pregnancy dating methodology. Furthermore, we found that total hCG differs according to maternal BMI, smoking, parity, ethnicity, child gender, placental weight and hyperemesis gravidarum symptoms. Our results suggest that the association between gestational age, hCG and fetal growth can cause less reliable ultrasound derived pregnancy dating, in particular in women with high or low levels of hCG. These data underline the complex relations between hCG, maternal and fetal factors, which should be taken into account when studying pregnancy complications. Our findings can serve as a reference for various clinical research studies and warrant further research on reference range determination for hCG during pregnancy.

APPENDIX

SUPPLEMENTAL TABLE 1. *Descriptive characteristics of the study population.*

	Median or N per group	(95% range)
hCG (IU/L)	35,403	(6,074– 100,351)
MoM	1.00	(0.33 – 2.46)
hCG z-scores (SD)	0.00	(-1.95 - +1.97)
Gestational age (weeks)		
Ultrasound	14.4	(10.1 – 26.1)
Last menstrual period[a]	12.4	(10.7 – 12.4)
Maternal age (years)	30.2	(19.3 - 39.1)
BMI (kg/m^2)	23.9	(18.7 – 36.5)
Placental weight (g)	620	(390 – 950)
Parity		
0	55.4	(4469)
1	30.0	(2417)
2	10.4	(834)
>2	4.2	(339)
Smoking		
Non-smokers	2874	(74.9)
Stopped smokers	381	(9.9)
Smokers	584	(15.2)
Education level		
Low	12.5	(1013)
Middle	47.2	(3805)
High	40.3	(3247)
Ethnicity		
Dutch	48.1	(3881)
Moroccan	7.3	(591)
Turkish	9.7	(779)
Surinamese	9.2	(745)
Other	25.7	(2069)
Child gender (boys %)	4062	(50.4)

[a] Available in a subset of 1517 women included during early pregnancy.
[b] Available in a subset of 5822 women.

SUPPLEMENTAL TABLE 2. *Gestational age specific, reference ranges for hCG in 7015 women with an uncomplicated pregnancy.*

Gestational week	N	Median	Minimum	2.5th	97.5th	Maximum
<9	27	59,973	455	2,305	93,158	142,584
9	40	78,705	22,655	22,906	129,808	129,909
10	91	73,369	16,464	29,794	133,550	145,910
11	221	61,794	16,151	23,806	133,221	187,852
12	678	56,692	8,105	23,807	114,394	144,919
13	1255	53,064	4,618	23,119	108,752	166,478
14	945	47,406	5,925	21,172	102,380	144,054
15	695	37,240	4,834	14,641	83,065	122,037
16	517	29,344	7,512	11,163	80,056	132,084
17	387	23,988	5,999	8,235	67,195	142,918
18	308	20,843	3,822	6,763	50,777	75,993
19	233	17,609	3,895	4,544	49,569	90,628
20	339	16,855	3,128	5,020	44,217	57,091
21	467	14,825	1,542	4,237	42,196	73,485
22	281	16,355	2,810	3,740	44,717	86,541
23	145	12,715	1,957	2,336	38,060	48,059
24	115	13,177	2,511	3,974	46,470	49,392
25	68	16,465	3,354	3,711	56,073	63,166
>25	203	13,503	518	2,224	53,749	74,719

hCG reference range values were calculated in women with an uncomplicated pregnancy, after exclusion of women with IVF treatment (N=38), twin pregnancy (N=90) or TOP pregnancies (N=2). Women that gave birth to a live-born, singleton pregnancy which was not premature (<37th week), small for gestational age (<2.5th gestational age specific birth weight), and of which the pregnancy was not complicated by pre-existing or pregnancy-induced hypertension or preeclampsia were considered uncomplicated. In addition, only the first recorded pregnancy within our study was used.

SUPPLEMENTAL TABLE 3. *Comparison of reference ranges of model-based versus non-parametric approach in 8065 women.*

Gestational week	N	Model median	Non-parametric median 95% CI		Model 2.5th	Non-parametric 2.5th 95% CI		Model 97.5th p	Non-parametric 97.5th 95% CI	
			Lower	*Upper*		*Lower*	*Upper*		*Lower*	*Upper*
9	50	69,204	64,445	86,851	27,702	22,655	34,928	151,958	115,695	129,909
10	106	70,180	69,350	81,789	29,323	16,080	35,517	149,276	124,030	163,393
11	255	63,848	58,665	67,776	27,358	16,372	27,634	133,260	111,836	160,438
12	790	56,681	54,420	58,079	24,478	19,136	24,382	117,604	110,101	126,782
13	1,418	52,152	50,969	53,938	22,231	21,744	25,231	109,276	103,691	116,644
14	1,069	47,015	45,492	48,706	19,231	17,801	21,972	101,601	96,478	110,567
15	800	37,478	36,055	39,115	14,314	12,719	15,322	85,173	78,800	89,593
16	594	30,220	28,491	30,887	10,630	9,881	12,134	72,860	72,645	94,288
17	455	25,106	23,409	26,182	8,178	7,232	9,485	63,878	63,839	75,868
18	354	20,937	19,022	23,441	6,432	5,116	7,526	55,455	47,556	57,687
19	271	18,379	16,315	19,764	5,417	3,998	6,321	50,064	41,000	64,266
20	389	17,014	16,500	18,487	4,837	4,027	6,368	47,480	39,201	47,446
21	530	15,429	13,838	16,282	4,189	3,379	4,689	44,380	38,682	47,894
22	330	14,714	14,955	17,383	3,768	3,411	5,119	43,961	38,271	56,344
23	165	13,084	10,829	15,145	3,133	2,153	3,332	40,805	34,138	55,899
24	134	13,879	11,862	15,978	3,100	2,934	4,297	45,210	35,997	48,845
25	79	14,047	11,753	18,961	2,933	3,354	4,598	47,684	36,803	63,166

SUPPLEMENTAL TABLE 4. *Mean hCG values according to characteristics of 8065 women.*

			Mean hCG (IU/L)		
	% or range	(N)	Univariate	Multivariate	MoM hCG*
Maternal age (years)					
1st quintile	15-25	(1630)	41,261	40,971	1.13
2nd quintile	25-29	(1623)	39,856	39,869	1.08
3rd quintile (ref)	29-32	(1616)	40,698	40,482	1.10
4th quintile	32-34	(1604)	39,539	39,604	1.09
5th quintile	34-46	(1591)	38,982[a]	39,461	1.10
Parity					
0 (ref)	55.4%	(4469)	41,310	40,852	1.13
1	30.0%	(2417)	39,004[c]	39,190[c]	1.08[c]
2	10.4%	(834)	37,431[c]	38,591[c]	1.04[c]
>2	4.2%	(339)	38,130[b]	39,958	1.07
Ethnicity					
Dutch (ref)	48.1%	(3881)	39,820	39,569	1.07
Moroccan	7.3%	(591)	41,817[a]	42,209[c]	1.18[c]
Turkish	9.7%	(779)	39,167	41,071[a]	1.14[c]
Surinamese	9.2%	(745)	42,155	42,603[b]	1.16[c]
Other	25.7%	(2069)	39,674	39,155	1.08
BMI					
1st quintile	15-21	(1626)	45,709[c]	45,545[c]	1.23[c]
2nd quintile	21-23	(1608)	41,260[a]	41,240	1.14
3rd quintile (ref)	23-25	(1604)	39,231	39,321	1.10
4th quintile	25-28	(1616)	38,109[a]	38,082[a]	1.05[a]
5th quintile	28-51	(1612)	36,053[c]	36,176[c]	0.96[c]
Smoking					
No (ref)	72.9%	(5880)	41,309	41,278	1.13
Former	8.3%	(673)	41,438	41,086	1.10
Yes	18.8%	(1512)	34,703[c]	34,979[c]	0.96[c]
Smoking dose#					
<5 cigarettes	48.3%	(919)	40,894	40,658	1.05
5-10 cigarettes	27.7%	(528)	36,695[b]	36,902	0.98
>10 cigarettes	24.0%	(457)	34,106[c]	34,815[c]	0.92[c]
Child Gender					
Boy	50.4%	(4062)	38,326	38,363	1.05
Girl	49.6%	(4003)	41,863[c]	41,825[c]	1.15[c]
Education level					
Low	12.5%	(1013)	38,622[a]	39,678[a]	1.11
Middle (ref)	47.2%	(3805)	39,821	40,249	1.10
High	40.3%	(3247)	40,841[c]	40,011	1.09

[a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$; MoM = Multiple of gestational age specific median; * multivariate analyses only. #Smoking dosage was determined by the maximum self-reported number of cigarettes smoked during pregnancy and was not imputated due to poor imputation quality. Values shown as mean hCG in IU/L or MoM value in all women after exclusion of women with IVF treatment and twin or TOP pregnancies. Univariate analyses were adjusted for gestational age at blood sampling. Multivariate analyses were adjusted for gestational age at blood sampling, maternal age, parity, smoking, maternal education level, ethnicity, BMI and child gender. hCG/MoM values were log transformed for statistical analyses.

SUPPLEMENTAL TABLE 5. *Mean hCG values according to characteristics of 8193 women from the Generation R study.*

	%	(N)	Mean hCG (IU/L) Univariate	Multivariate	MoM hCG*
Fertility treatment					
No	98.7%	(7996)	39,287	40,083	1.10
IVF	0.5%	(38)	47,501[b]	47,740[a]	1.30[a]
IUI	0.1%	(11)	39,342	41,058	1.08
Ovulation induction ICSI or other	0.7%	(58)	38,898	37,440	1.03
Twins					
No	99.0%	(8065)	40,099	40,096	1.10
Yes	1.0%	(82)	67,488[c]	67,853[c]	1.83[c]

[a] *P*<0.05; [b] *P*<0.01; [c] *P*<0.001; MoM = Multiple of gestational age specific median; * multivariate analyses only; Values shown as mean hCG in IU/L or MoM value in all women after exclusion of women with a TPO pregnancy and additionally IVF treatment or twin pregnancy, also, 8 women pregnant with twins after IVF treatment were excluded. Univariate analyses were adjusted for gestational age at blood sampling. Multivariate analyses were adjusted for gestational age at blood sampling, maternal age, parity, smoking, maternal education level, ethnicity, BMI and child gender. hCG/MoM values were log transformed for statistical analyses.

SUPPLEMENTAL TABLE 6. *Mean MoM hCG values according to self-reported episodes of hyperemesis gravidarum symptoms.*

	% or range	(N)	Mean MoM hCG Univariate	Multivariate
Self-reported episodes of weekly				
Reflux/belching				
Never (ref)	44.6%	(2998)	1.08	1.08
Less than once	15.1%	(1017)	1.07	1.06
Once	9.2%	(622)	1.10	1.10
Few times	17.8%	(1198)	1.12[a]	1.12[b]
Daily	13.2%	(890)	1.16[c]	1.16[c]
Linear β (SE)			0.017 (0.004); P<0.01	0.020 (0.004); P<0.01
Nausea				
Never (ref)	18.5%	(1263)	1.03	1.05
Less than once	12.8%	(871)	1.09[b]	1.08
Once	8.2%	(557)	1.05	1.05
Few times	27.2%	(1854)	1.09[b]	1.09[b]
Daily	33.4%	(2279)	1.15[c]	1.14[c]
Linear β (SE)			0.024 (0.004); P<0.01	0.021 (0.004); P=0.01
Vomiting				
Never (ref)	55.2%	(3696)	1.08	1.08
Less than once	14.8%	(991)	1.09	1.08
Once	6.2%	(418)	1.10	1.10
Few times	13.2%	(886)	1.14[b]	1.14[b]
Daily	10.5%	(706)	1.13[a]	1.14[b]
Linear β (SE)			0.015 (0.004); P<0.01	0.017 (0.004); P<0.01

[a] *P*<0.05 ; [b] *P*<0.01; [c] *P*<0.001 ; MoM = Multiple of gestational age specific median. Values shown as mean MoM values in all women after exclusion of women with IVF treatment or twin pregnancy. Self-reported hyperemesis gravidarum symptoms were not imputated due to the large number of missing values. Univariate analyses were adjusted for gestational age at blood sampling. Multivariate analyses were adjusted for gestational age at blood sampling, maternal age, parity, smoking, maternal education level, ethnicity, BMI and child gender. MoM values were log transformed for statistical analyses.

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CHAPTER 6

STIMULATION OF THYROID FUNCTION BY HCG DURING PREGNANCY: A RISK FACTOR FOR THYROID DISEASE AND A MECHANISM FOR KNOWN RISK FACTORS

Tim I.M. Korevaar, Yolanda B. de Rijke, Layal Chaker, Marco Medici, Vincent W. V. Jaddoe, Eric A.P. Steegers, Theo J. Visser and Robin P. Peeters

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ABSTRACT

BACKGROUND Thyroid autoimmunity is a major risk factor for gestational thyroid disease and recently various other risk factors have been identified, including maternal age, BMI and parity. hCG is an important determinant of gestational thyroid function, yet it is unknown to what extent differences in hCG concentration affect the risk for thyroid disease. We have recently shown that TPOAb positivity impairs the thyroidal response to hCG stimulation, which may suggest that this is a mechanism through which thyroid autoimmunity acts as a risk factor for thyroid disease.

OBJECTIVE To study if hCG is a risk factor for thyroid disease entities, and if recently identified risk factors for thyroid disease may influence the thyroidal response to hCG stimulation.

METHODS hCG, TSH and FT4 were measured in 5435 pregnant women participating in a prospective cohort. The association of hCG with thyroid disease entities, and the association of known risk factors with thyroidal response to hCG stimulation were studied using multivariable linear regression models.

RESULTS Higher hCG concentrations were associated with a higher risk of subclinical and overt hyperthyroidism. Lower hCG concentrations were associated with a higher risk of hypothyroxinemia. In contrast, hCG concentrations were not associated with subclinical hypothyroidism. Further analyses showed that in women with hypothyroxinemia, high hCG concentrations still suppressed TSH. However, in women with subclinical hypothyroidism, high hCG concentrations were not associated with higher FT4.

Higher BMI, male fetal sex and maternal parity >2 were associated with a lower thyroidal response to hCG stimulation.

CONCLUSIONS hCG is associated with the risk of (subclinical) hyperthyroidism and hypothyroxinemia, but not with the risk of (subclinical) hypothyroidism. Women with hypothyroxinemia have a normal response to thyroidal stimulation by hCG, but this was abnormal in women with subclinical hypothyroidism. Known risk factors for thyroid dysfunction (BMI and parity), and also male fetal sex, are associated with a lower thyroidal response to hCG stimulation.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a pregnancy specific hormone that exerts thyrotropic activity via its affinity for the TSH receptor.[1] High hCG concentrations during early pregnancy effectuate an increase in FT4 concentrations which subsequently leads to a decrease in TSH concentrations.[1,2] The trajectory of hCG encompasses a swift rise after conception and peak concentrations are reached near the end of the first trimester.[1,3] Subsequently, concentrations decrease until they reach a stable concentration around the 20th week of pregnancy.[1,3] It is believed that the thyrotropic activity of hCG is important during early pregnancy to facilitate the increased energy expenditure and metabolic demand while it also safeguards sufficient thyroxine availability for the developing fetus.[4,5] In order to identify women with an abnormal gestational thyroid function, recommendations of international guidelines incorporate hCG-specific alterations in thyroid physiology (for example by recommending pregnancy-specific reference ranges) as well as risk factors for thyroid disease.[6-8]

Overt thyroid disease is a well-known risk factor for various adverse pregnancy and/or child outcomes.[9-11] Although a fast growing body of evidence now suggests that also milder forms, such as subclinical hypothyroidism and hypothyroxinemia, also increase the risk of adverse outcomes, the underlying pathogenic mechanism for these disease entities remains to be elucidated.[9,12] Since thyroid disease entities are defined by the absolute levels of TSH and FT4 concentrations, it is important to note that any determinant of thyroid function can be considered as a risk factor for thyroid dysfunction. Thyroid autoimmunity, in most studies reflected by thyroperoxidase antibody (TPOAb) positivity, is the most important risk factor for thyroid disease.[13] However, recent studies indicate that other characteristics such as maternal BMI, age, smoking, parity, ethnicity and urinary iodine excretion, are also risk factors for thyroid disease during pregnancy.[14-20] Although it is well-known that hCG is an important stimulator of thyroid function during pregnancy, it is currently unknown if, and to what extent, the hCG concentration at time of blood sampling is a risk factor for thyroid disease.[1,2,21]

In addition, both low and high hCG concentrations are associated with adverse outcomes that have also been associated with an abnormal thyroid function.[22-26] Therefore, it is possible that abnormal hCG concentrations underlie the association of thyroid function with adverse outcomes, or abnormal hCG concentrations subsequently cause thyroid dysfunction which may mediate the adverse effects.

We have previously shown that TPOAb positivity is associated with a severely impaired thyroidal response to hCG stimulation.[27] This suggests that an impaired response to hCG stimulation may be a mechanism through which thyroid autoimmunity increases the risk of thyroid dysfunction during pregnancy. However, it is unknown to what extent other risk factors may attenuate the thyroidal response to hCG stimulation and whether this is a relevant mechanism through which these risk factors increase the risk of thyroid disease during pregnancy.

This study aims to identify to what extent the hCG concentration at blood sampling is a risk factor for thyroid disease, and also if more recently identified risk factors for thyroid disease during pregnancy may impair the thyroidal response to hCG stimulation.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[28] hCG and TSH, FT4 or TPOAb measurements were available for 5707 pregnant women. Women with twin pregnancies (N=128), pre-existing thyroid

disease (N=73), thyroid (interfering) medication usage (N=4), fertility treatment (N=67) were excluded. The general design, all research aims and the specific measurements in the Generation R Study have been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam and written informed consent was available for all participants.[28]

Biochemical measurements

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks; range 9.6-17.6). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden). TPOAb concentrations were regarded as positive if >60 IU/ml. Euthyroidism and thyroid disease entities were defined according to pre-defined population-based cut-off values using the 2.5th and 97.5th percentiles in TPOAb negative women.[29] Additionally, in order to achieve adequate statistical power, overt hypothyroidism was also defined by the 5th-95th percentile cut-offs (assuming a similar effect estimate as for the proportion of women with overt hyperthyroidism above versus below the hCG median, N=50 women with overt hypothyroidism would be required to achieve alpha=0.05 and power=80%). Human chorionic gonadotropin was analyzed in serum using a solid-phase two-site chemiluminescent immunometric assay, calibrated against WHO 3rd IS 75/537, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The Siemens assay gives measurement of total hCG and detects serum hCG, hyperglycosylated hCG, serum nicked hCG, serum nicked hCG missing the β-subunit C-terminal peptide, serum nicked hyperglycosylated hCG, serum asialo hCG, serum hCG-β and serum nicked hCG β.[30] The inter assay coefficient of variation was 8.0, 6.3 and 5.1% at the concentration of 9.7, 53.1 and 821.5 IU/L , respectively.[3] Iodine and creatinine were measured in spot urine samples of a subset of women (N=1986) in samples collected at the same time as serum. The urinary iodine/creatinine ratio was calculated and used for analyses, further details have been described elsewhere.[31] As we determined previously, this study population is iodine sufficient.[31]

Based on the literature and data availability, we selected known risk factors for thyroid disease or determinants of hCG as potential determinants of the thyroidal response to hCG stimulation. These included maternal age, BMI, smoking, ethnicity, parity, fetal sex and urinary iodine excretion.[3,14-20] Information on maternal age, smoking status and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands.[28] Ethnicity was categorized according to the four major ethnic groups in the Netherlands (Dutch, Moroccan, Turkish and Surinamese) and a Western or non-Western group, details on which have been described previously.[18] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). We have previously shown that TPOAb positive women have a severely impaired thyroidal response to hCG stimulation.[27] Therefore, TPOAb positive women were excluded from analyses on the course of hCG, FT4 and TSH and also on analyses on determinants of hCG mediated thyroid stimulation. In addition, we have previously shown that placental angiogenic factors (soluble FMS-like tyrosine kinase (sFlt1) and placental growth factor (PlGF)) are determinants of maternal thyroid function during pregnancy and therefore we also additionally adjusted analyses for these factors.[32]

Covariates

Information on maternal education level was obtained by questionnaires during pregnancy and was defined as low (non/primary), middle (secondary) or high (higher education). Information on fertility treatment, and the sex of the child were obtained from community midwives, obstetricians, and hospital registries.

Statistical analysis

To fulfill model assumptions and acquire optimal fit of regression models, TSH and FT4 concentrations were log transformed and models were fitted using restricted cubic splines with 3 or 4 knots. The course of hCG, TSH and FT4 during early pregnancy was plotted by calculating the median concentrations per week of pregnancy. The association of hCG with the risk of thyroid disease entities was investigated using multivariable logistic regression models. We investigated determinants of hCG mediated thyroid stimulation by using multivariable linear regression models with a product interaction term of each variable of interest with hCG. Due to known constraints of statistical power for interaction analyses, a *P*-value<0.15 was considered statistical significance of interaction terms and analyses were subsequently stratified to assess (clinical) relevance. In order to investigate the role of thyroidal hCG stimulation in different disease entities we investigated the association of hCG with FT4 in women with subclinical hypothyroidism and subclinical hyperthyroidism (because opposite to TSH, there is still large variation in FT4 concentrations per definition of the diagnosis), and the association of hCG with TSH in women with hypothyroxinemia (because opposite to FT4, there is still large variations in TSH concentrations per definition of the diagnosis). In order to investigate this we selected euthyroid TPOAb negative women (and women with one of the subclinical disease entities) and introduced a product interaction term of the clinical disease entity (as a binary variable) with hCG into the linear regression model.

For covariates with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used. Five imputed data sets were created and pooled for analyses. Smoking, education level, ethnicity, parity and BMI were added to the model (missing due to non-response/non-recording in 13.1%, 7.2%, 4.1%, and <1.0%, respectively). Furthermore, we added hCG, TSH, FT4 and TPOAb concentrations, gestational age at blood sampling, maternal age and fetal gender as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. For clarity, nulliparous and primiparous were combined into a single category because the slope was similar and figures better displayed associations with 3 categories. All statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA) or R statistical software version 3.03 (package *rms* and *visreg*) and a *P*-value <0.05 was considered for statistical significance.

RESULTS

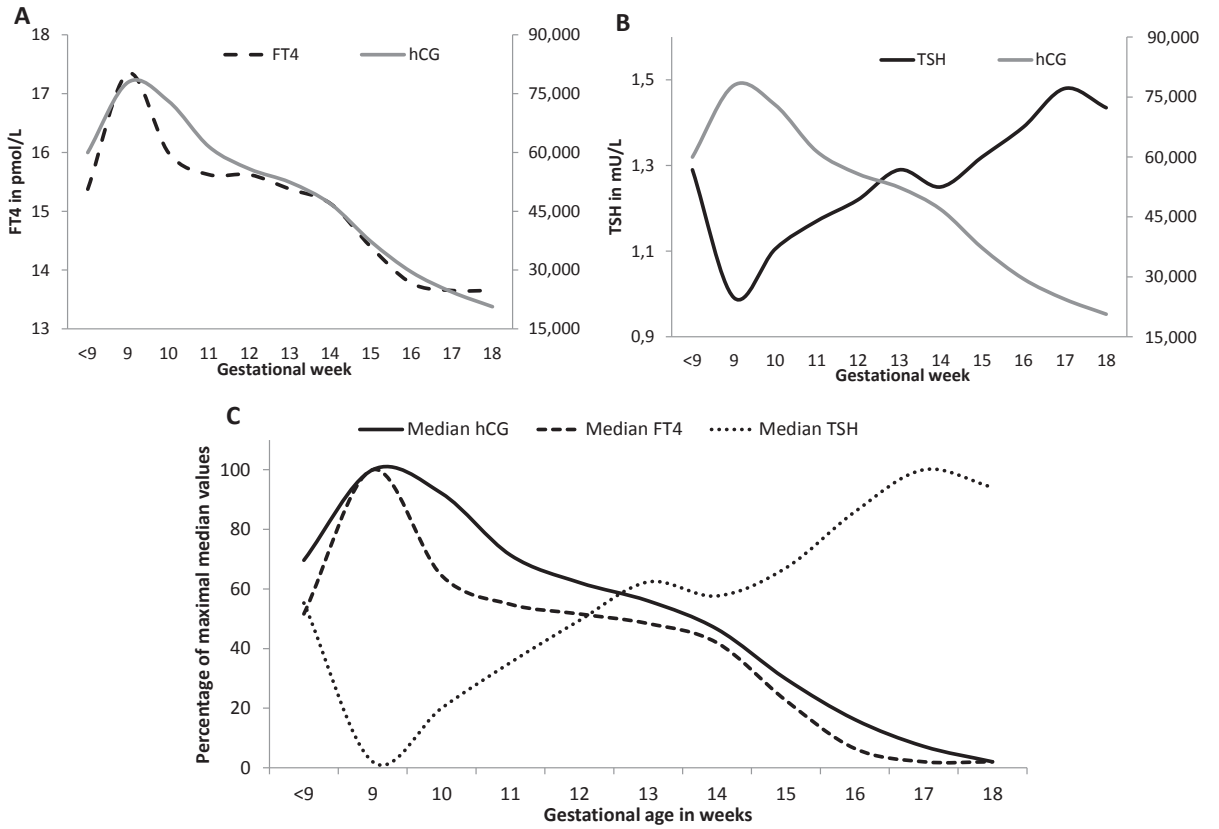
The final study population comprised 5435 women that were predominantly nulliparous or primiparous (86.9%), non-smokers (73.5%) and of Dutch origin (51.6%; Table 1). Compared to euthyroid women, mean BMI was higher in women with subclinical hypothyroidism (+0.63 kg/m^2; *P*=0.045) and in women with hypothyroxinemia (+2.46 kg/m^2; *P*<0.001) but not for women with subclinical hyperthyroidism (-0.42 kg/m^2; *P*=0.39). Median hCG concentrations swiftly increased to peak values around the 9th week and then steadily decreased until the 18th week of pregnancy (Figure 1A, B, C). Throughout early pregnancy, the course of median FT4 concentrations mimicked that of hCG concentrations (Figure 1A, C), while the course of TSH concentrations mirrored the course of hCG concentrations (Figure 1B, C).

TABLE 1. Descriptive statistics of 5435 women.

		Median	(95% range)
Median TSH	(mU/L)	1.35	(0.04-4.55)
Median FT4	(pmol/L)	14.8	(10.3-22.3)
Median T4	(nmol/L)	145	(95-223)
Median hCG	(IU/L)	44,416	(11,989-107,273)
Overt hypothyroidism	(n(%))	19	(0.03)
Subclinical hypothyroidism*	(n(%))	181	(3.3)
Hypothyroxinemia	(n(%))	141	(2.6)
Overt hyperthyroidism	(n(%))	53	(1.0)
Subclinical hyperthyroidism	(n(%))	75	(1.4)
TPOAb positivity	(n(%))	286	(5.3)
Gestational age[a]		13.2	(9.6-17.6)
Maternal age[d]		30.3	(19.5-38.8)
BMI		23.6	(18.5-35.8)
Parity [c]			
0		3095	(56.9)
1		1633	(30.0)
2		508	(9.3)
>2		199	(3.7)
Smoking [c,e]			
Non-smokers		3997	(73.5)
Stopped smokers		483	(8.9)
Smokers		955	(17.6)
Education level [e]			
Low		568	(10.5)
Middle		2510	(46.1)
High		2357	(43.4)
Ethnicity [c,e]			
Dutch		2803	(51.6)
Moroccan		341	(6.3)
Turkish		455	(8.4)
Surinamese		479	(8.8)
Other western		491	(9.0)
Other non-western		866	(15.9)
Child gender[c] (boys %)		2752	(50.6)

** After exclusion of TPOAb positive women N=118 (2.3%)*
[a] At time of blood sampling; Data shown as median in weeks
[b] Data shown as mean in grams (SD)
[c] Data shown as n (%)
[d] Data shown as median in years
[e] Data shown after imputation of missing data (13.1% for smoking, 7.2% for education level, 4.1% for ethnicity and <1.0% for BMI and parity).

FIGURE 1. Course of hCG, TSH and FT4 during early pregnancy in TPOAb negative women.



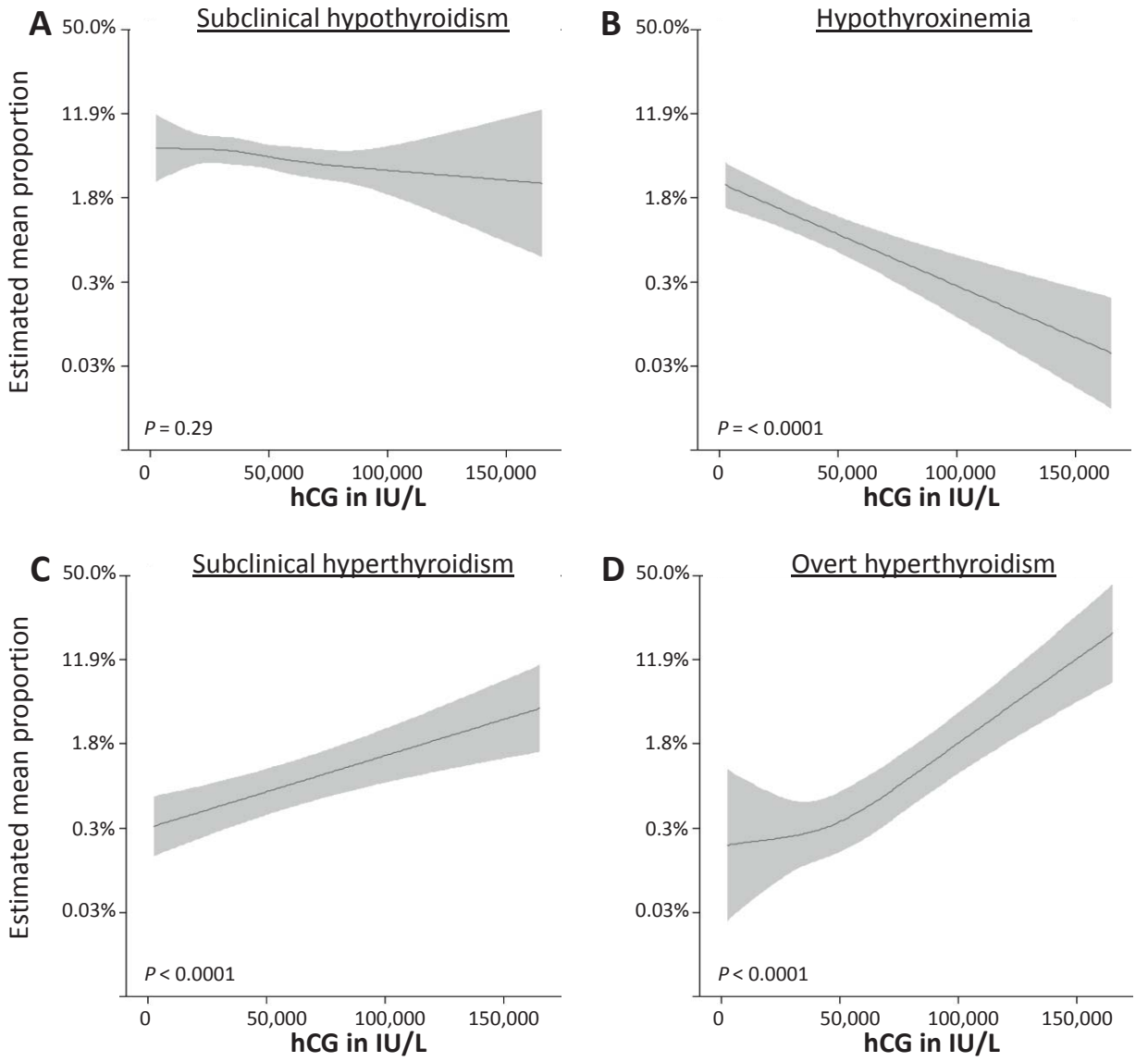
Data is shown as median levels per gestational week after exclusion of TPOAb positive women.

The association of hCG concentrations with thyroid disease

There was a positive association of hCG concentrations with subclinical and overt hyperthyroidism (both *P*<0.001); Figure 2C, D). There was a negative association of hCG concentrations with hypothyroxinemia (*P*<0.001; Figure 2B). In contrast, there was no association of hCG concentrations with overt hypothyroidism, also when overt hypothyroidism was defined according to 5th and 95th percentile cut-offs range (N=19 and N=54, respectively; *P*>0.22; Supplemental Figure 1A, B). Similarly, hCG concentrations were not associated with the risk of subclinical hypothyroidism (*P*=0.29; Figure 2A), regardless of TPOAb status (*P*=0.88; Supplemental Figure 1C). The association of hCG concentrations with other disease entities did not change after exclusion of TPOAb positive women.

Because little is known on the mechanisms underlying the biochemical discrepancies of subclinical hypothyroidism and hypothyroxinemia, we subsequently investigated the thyroidal stimulation by hCG within these two groups. We studied the association of hCG concentrations with TSH concentrations in women with hypothyroxinemia, and of hCG concentrations with FT4 concentrations in subclinical hypothyroidism (because the definition of both disease entities do not allow to study variation of FT4 or TSH, respectively) and compared this to euthyroid women. In women with hypothyroxinemia, the association of hCG concentrations with TSH concentrations was similar to euthyroid women (*P for difference with euthyroid* = 0.72, Figure 3B). However, in women with subclinical hypothyroidism, the association of hCG concentrations with FT4 concentrations was considerably attenuated compared to euthyroid women (*P for difference with euthyroid* = 0.047; Figure 3A). All results remained similar after exclusion of TPOAb positive women (data not shown).

FIGURE 2. The association between hCG and the risk of (subclinical) thyroid disease entities.



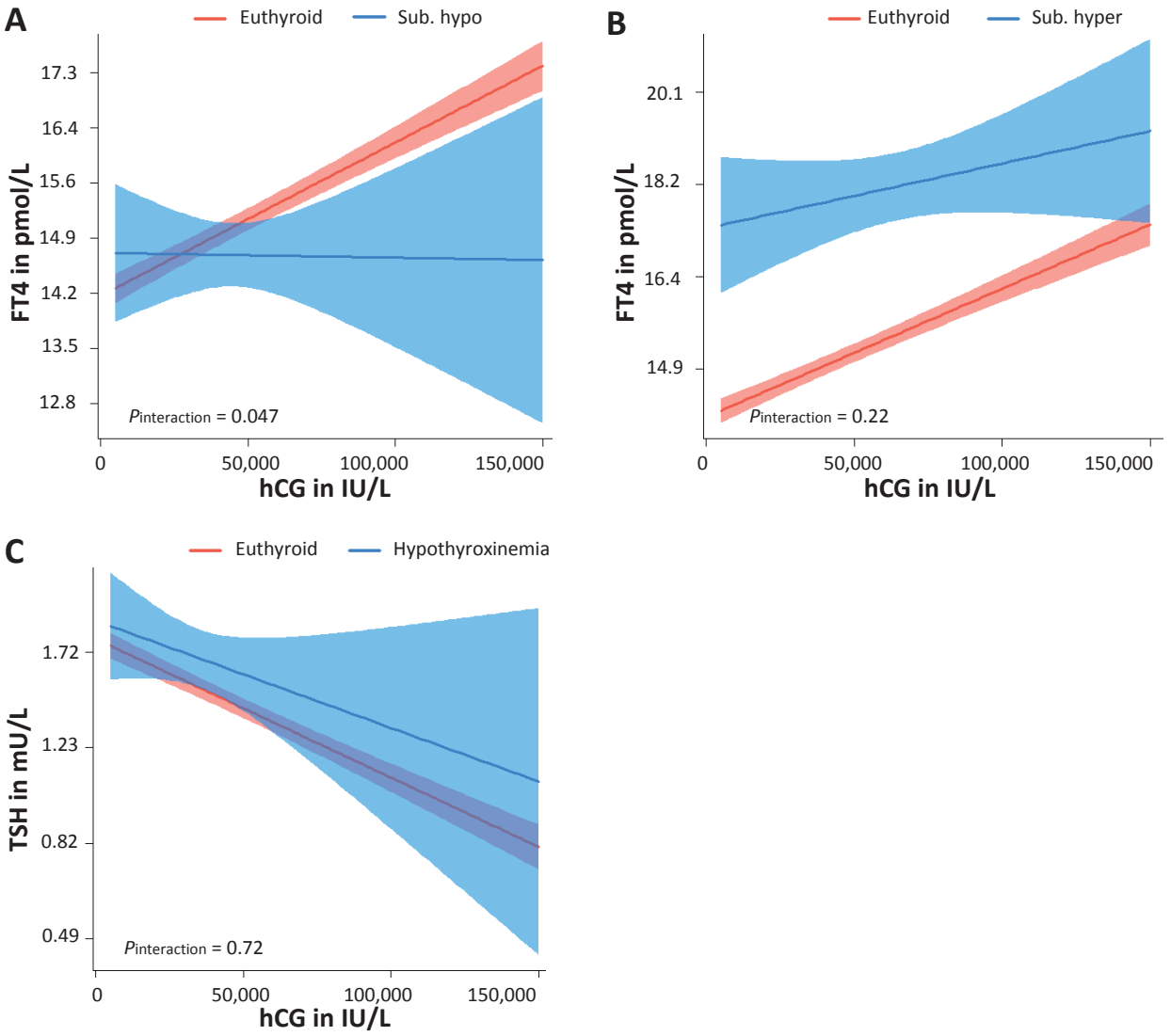
Figures are shown as mean proportion (black line) with 95% confidence interval (grey area). All analyses were adjusted for maternal age, BMI, smoking, parity, education level, ethnicity and fetal gender. Depicted analyses were performed in the full dataset, not excluding TPOAb positive women.

Thyroidal response to hCG stimulation according to known risk factors

Overall, a higher BMI was associated with higher TSH and lower FT4 concentrations. However, this was amplified at higher hCG concentrations, indicating that a higher BMI was associated with a lower thyroidal response to hCG stimulation (*P for difference*= 0.003 and 0.068; Figure 4A, B). Although there were no fetal sex differences in TSH and FT4 at low hCG concentrations, male fetal sex was associated with a lower thyroidal response to hCG stimulation, since women pregnant of a male fetus had higher TSH and lower FT4 concentrations when hCG concentrations were high (*P for difference*= 0.0064 and 0.0006; Figure 4C, D). A high parity was associated with a lower thyroidal response to hCG stimulation (*P for difference*= 0.019 and 0.036; Figure 4E, F).

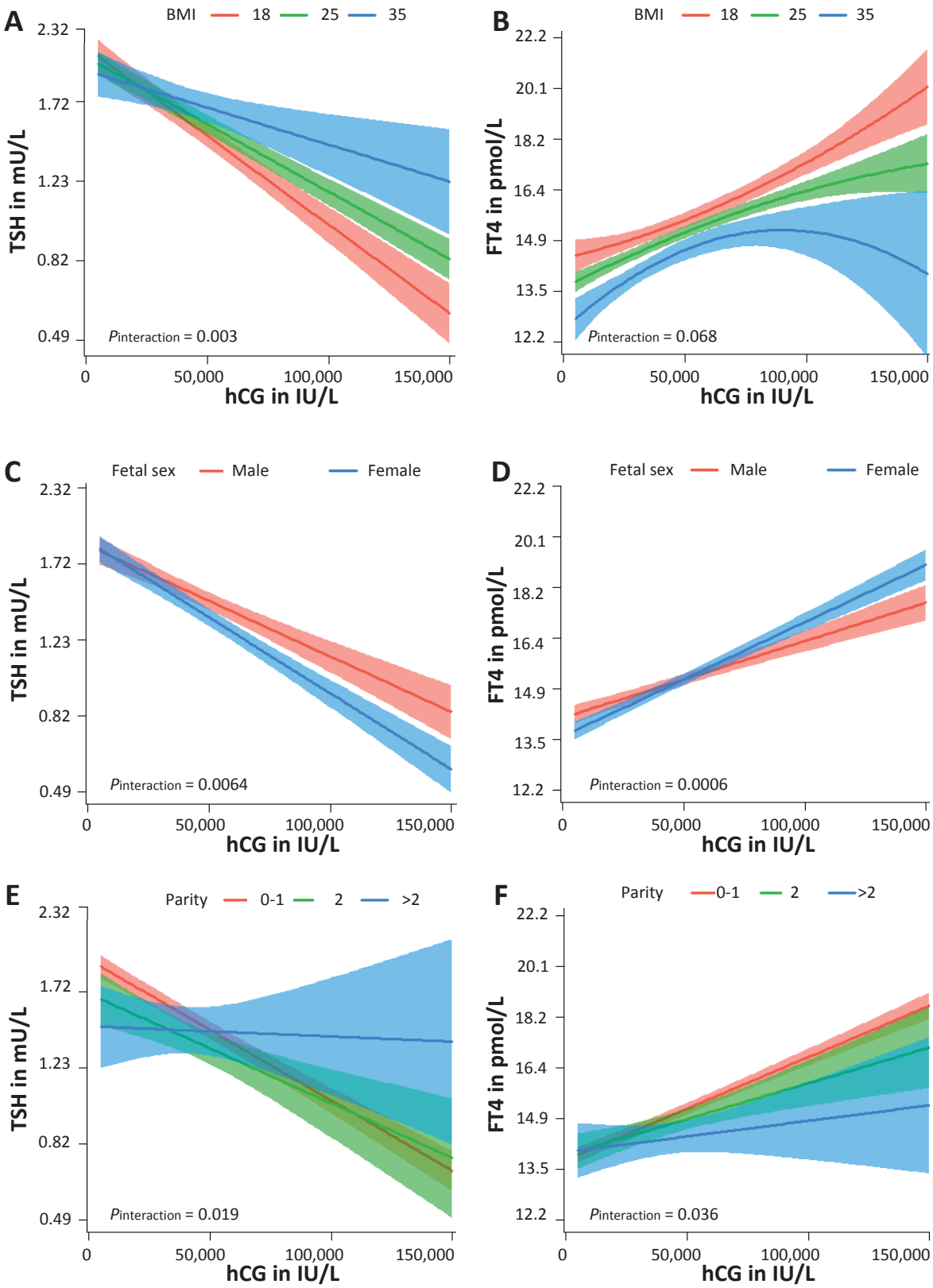
There were no consistent differences in thyroidal response to hCG stimulation according to maternal ethnicity, age, smoking or iodine status (data not shown). All results remained similar after additional adjustment for angiogenic factors (soluble FMS-like tyrosine kinase (sFlt1) and placental growth factor (PlGF); data not shown).

FIGURE 3. hCG mediated gestational thyroid stimulation in subclinical disease.



Figures are shown as estimated mean values (lines) and 95% confidence intervals (colored area) for women with subclinical hypothyroidism (A, blue line), subclinical hyperthyroidism (B, blue line), hypothyroxinemia (C, blue line) in comparison to euthyroid women (A,B,C, red line). All analyses were adjusted for maternal age, smoking, ethnicity, education level, parity and fetal gender. Depicted analyses were performed after excluding TPOAb positive women.

FIGURE 4. Differences in hCG mediated gestational thyroid stimulation.



Figures are shown as estimated mean values (lines) and 95% confidence intervals (colored area) for women with a BMI of 18, 25, or 35 kg/m2 (A, B; red, green or blue line respectively), women with a male or female fetal fetus (C,D; red or blue line, respectively) and women with a parity of 0-1, 2 or >2 (E,F; red, green or blue line, respectively). All analyses were adjusted for maternal age, smoking, ethnicity, education level, and where relevant BMI, parity and fetal gender. Depicted analyses were performed after excluding TPOAb positive women.

DISCUSSION

Since the seminal paper by Glinoer *et al*. (1990) that reported on the association of hCG concentrations with TSH and FT4 concentrations, only few studies have investigated the effects of hCG concentrations on thyroid function during pregnancy.[1,2,21] However, accumulating evidence since 1990 has shown that subclinical thyroid disease is associated with a higher risk of adverse outcomes pregnancy and child outcomes.[8,9] In the current study we identify differences in the association of hCG concentrations with thyroid disease entities. We demonstrate that high hCG concentrations are associated with a lower risk of hypothyroxinemia and a higher risk of subclinical and overt hyperthyroidism while hCG concentrations are not associated with the risk of overt or subclinical hypothyroidism. In line with these results we also show that the association of hCG concentrations with thyroid function in women with hypothyroxinemia and subclinical hyperthyroidism is similar to euthyroid women, while it was considerably attenuated in women with subclinical hypothyroidism. Furthermore, we show that higher BMI, male fetal sex and parity >2 are associated with a lower thyroidal response to hCG stimulation.

Within a self-proclaimed healthy population, practically all women with the biochemical phenotype of overt hyperthyroidism according to the recommendations of international guidelines will have transient gestational hyperthyroidism, or thyrotoxicosis, due to high hCG concentrations.[6-8,33] In the current study, we show that hCG concentrations are indeed a major determinant of gestational hyperthyroidism. These numbers confirm the current belief that gestational hyperthyroidism should be considered as a mild thyroid function abnormality, unlikely to lead to adverse outcomes or require antithyroid drug treatment.[33,34] However, because our results indicate that even very high hCG concentrations do not account for all cases of gestational hyperthyroidism, further studies are warranted to investigate the existence of potential sub-phenotypes.[35]

Hypothyroxinemia is associated with an increased risk of adverse offspring outcomes, in particular neurocognition,[36-39] but the pathogenic mechanism for hypothyroxinemia remains unclear. Although hypothyroxinemia was for long considered as a pregnancy-specific disease entity that reflects a state of mild iodine deficiency, this is challenged by the occurrence of hypothyroxinemia in iodine sufficient areas as well as the lack of (F)T4 increase following iodine supplementation.[40-45] The current study, performed in an iodine sufficient population, is the first study to demonstrate that low hCG concentrations at blood measurement are a risk factor for hypothyroxinemia. These results suggest that hypothyroxinemia in iodine sufficient areas is caused, at least partially, by low hCG concentrations and confirms that hypothyroxinemia is a pregnancy-specific disease entity. Subsequently, we also show that in women with hypothyroxinemia, the negative association of hCG with TSH is similar to euthyroid women. Given that the association of hCG concentrations with TSH is predominantly mediated via a hCG-mediated increase in FT4 concentrations, this suggests that the functional capacity of the thyroid is not impaired in women with hypothyroxinemia. In other words, it is likely that a pregnancy-specific increase in FT4 concentrations in hypothyroxinemic women is similar to euthyroid women. These findings may add to the scientific debate on the cause of hypothyroxinemia. In addition, it can be speculated that women with hypothyroxinemia are more sensitive to thyroid hormone at the level of the hypothalamus and/or the pituitary, and that this adds to their biochemical phenotype of low FT4 with high TSH concentrations. Further studies are needed to investigate non-gestational thyroid function of women with hypothyroxinemia during pregnancy and the effects of hCG in populations with (moderately) deficient iodine status.

This is the first study to demonstrate that serum hCG concentration are not associated with the risk of subclinical hypothyroidism, suggesting a lack of thyroidal response to hCG stimulation in women with subclinical hypothyroidism during pregnancy. In women with a lower thyroid functional capacity the

hCG mediated increase in FT4 concentrations may be impaired, which may lead to a lack of decrease in TSH concentrations during pregnancy. This is also supported by our finding that high hCG concentrations were not associated with higher FT4 concentrations within the group of women with subclinical hypothyroidism. On top of the lack of decrease in TSH concentrations, we speculate that women with a lower thyroid functional capacity may already have high-normal TSH concentrations going into pregnancy. Together, this may lead to the biochemical phenotype of subclinical hypothyroidism.

Several factors may contribute to an impaired thyroidal response to hCG. It is reported that approximately one-third of all women with subclinical hypothyroidism are TPOAb positive.[46] We have previously shown that the thyroidal response to hCG stimulation is severely impaired in the majority of TPOAb positive women.[27] However, the results in this study all remained similar after exclusion of TPOAb positive women. Although women with subclinical hypothyroidism had a higher mean BMI, women with hypothyroxinemia had an even higher mean BMI but still a hCG mediated FT4 response similar to euthyroid women and all analyses were adjusted to BMI. This suggests that the lower thyroid functional capacity in women with subclinical hypothyroidism is not due to thyroid autoimmunity or a higher BMI. Future studies are needed in order to investigate the underlying cause of a lower thyroid functional capacity in women with subclinical hypothyroidism.

Various studies have shown that a high BMI is associated with higher TSH and lower FT4 concentrations.[47] Most likely the effects on TSH are mediated via higher leptin levels, increasing thyrotropin releasing hormone via up-regulation of proTRH gene expression and by increasing the conversion of proTRH to mature TRH.[48,49] Additionally, higher BMI may be associated with lower FT4 through increase T4 binding or increased FT4 assay interference, as higher body fat mass is associated with higher TBG concentrations.[50] We now demonstrate that a higher BMI is also associated with a lower thyroidal response to hCG stimulation which might suggest that a higher BMI leads to a lower thyroid functional capacity or towards the presence of an underlying factor that is associated with both BMI and the thyroid functional capacity. Based on the results from the current study, gestational FT4 and TSH concentrations in overweight or obese women to a lesser extent reflect changes in hCG concentrations than in normal-weight women.

We show that there are small, but consistent fetal sex specific differences in the thyroidal response to hCG stimulation. Fetal sex specific differences have been shown for various outcomes including placental gene expression, placentation markers, disease symptomatology and risk and severity of adverse pregnancy outcomes.[51-53] Although our results might be confounded by fetal sex specific differences in placental angiogenic factors (such as sFlt1 and PlGF)[32,53] additional adjustment for sFlt1 and PlGF did not change the results. Alternatively, as hCG isoforms have a different stimulating potential for the TSH receptor,[54-56] fetal sex specific differences in hCG isoforms could explain fetal-sex specific differences observed in the current study.

Iodine is an important determinant of thyroid function and both low and high concentrations of urinary iodine to creatinine ratio (UICr) have been associated with lower thyroid function.[20] In this study, we did not find differences in thyroidal response to hCG stimulation between groups with a different iodine status based on UICr. However, our cohort is iodine sufficient and therefore we could not properly investigate the difference in thyroidal response to hCG according to low concentrations of iodine.

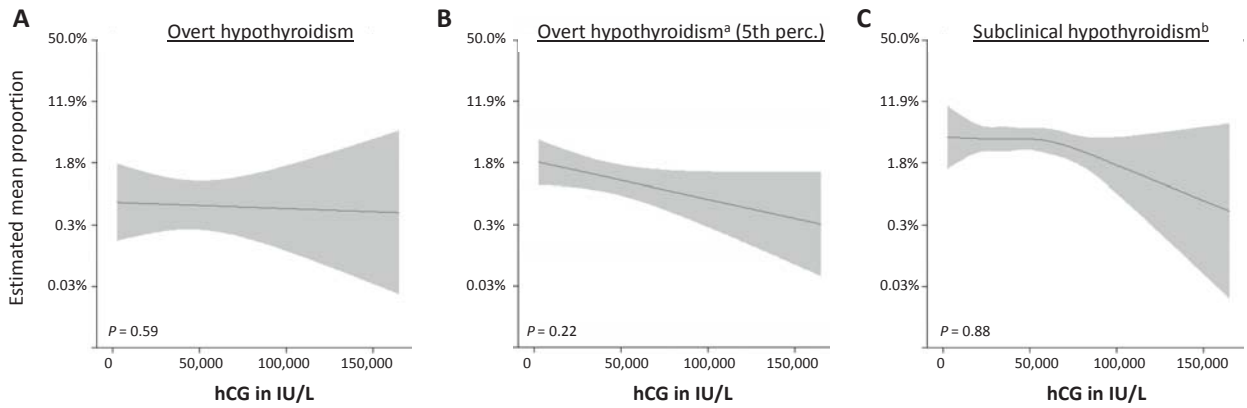
To our knowledge, this is the first study that investigated the association of differences in hCG concentrations with the risk of (subclinical) disease entities and the thyroidal response to hCG stimulation. We were able to study this in a large, prospective cohort with detailed phenotype data. We were limited by the fact that only a single measurement of hCG concentrations and thyroid function was available. However, thyroid function measurements are highly correlated throughout pregnancy.[57] Furthermore, in clinical practice, decisions are based on the interpretation of a single measurement

which mimics the data availability in this study. We were also limited by the relative low number of women with overt hypothyroidism which did not allow for adequately powered analyses. However, an alternative definition for hypothyroidism according to the highest and lowest 5th percentiles did not change the results. In addition, inadequate statistical power may also account for the lack of association between hCG concentrations and subclinical hypothyroidism. However, the number of women with subclinical hypothyroidism was equal or higher than other disease entities, suggesting that the potential effect of hCG concentrations on subclinical hypothyroidism would be much smaller. Another potential limitation is the fact that we were not able to measure the various isoforms of hCG, that may exert a different stimulation of the TSH receptor. However, changes in the ratio of hCG isoforms predominantly occur in the first 6 weeks of pregnancy, which is well before women were included in this study.[58]

In conclusion, we show that hCG concentrations are a determinant of hypothyroxinemia and subclinical or overt hyperthyroidism. hCG concentrations are not a determinant of subclinical hypothyroidism, and women with subclinical hypothyroidism have an impaired FT4 response to hCG stimulation. We demonstrate that some, but not all, risk factors for thyroid disease in pregnancy (higher BMI, parity) as well as male fetal sex are determinants of the thyroidal response to hCG stimulation. These data give novel insights into the (patho)physiology of thyroid disease during pregnancy. Future studies are needed to determine to what extent differences in thyroidal response are associated with adverse pregnancy outcomes or offspring development.

APPENDIX

SUPPLEMENTAL FIGURE 1. The association between hCG and the risk of (subclinical) thyroid disease entities.



[a] This disease entitiy was defined according to 5th percentile cut-offs for TSH and FT4 to exert more statistical power.

[b] This analysis was performed in TPOAb negative women only.

Figures are shown as mean risk (black line) with 95% confidence interval (grey area). All analyses were adjusted for gestational age at blood sampling. Y-axis values are backtransformed log(odds) values .

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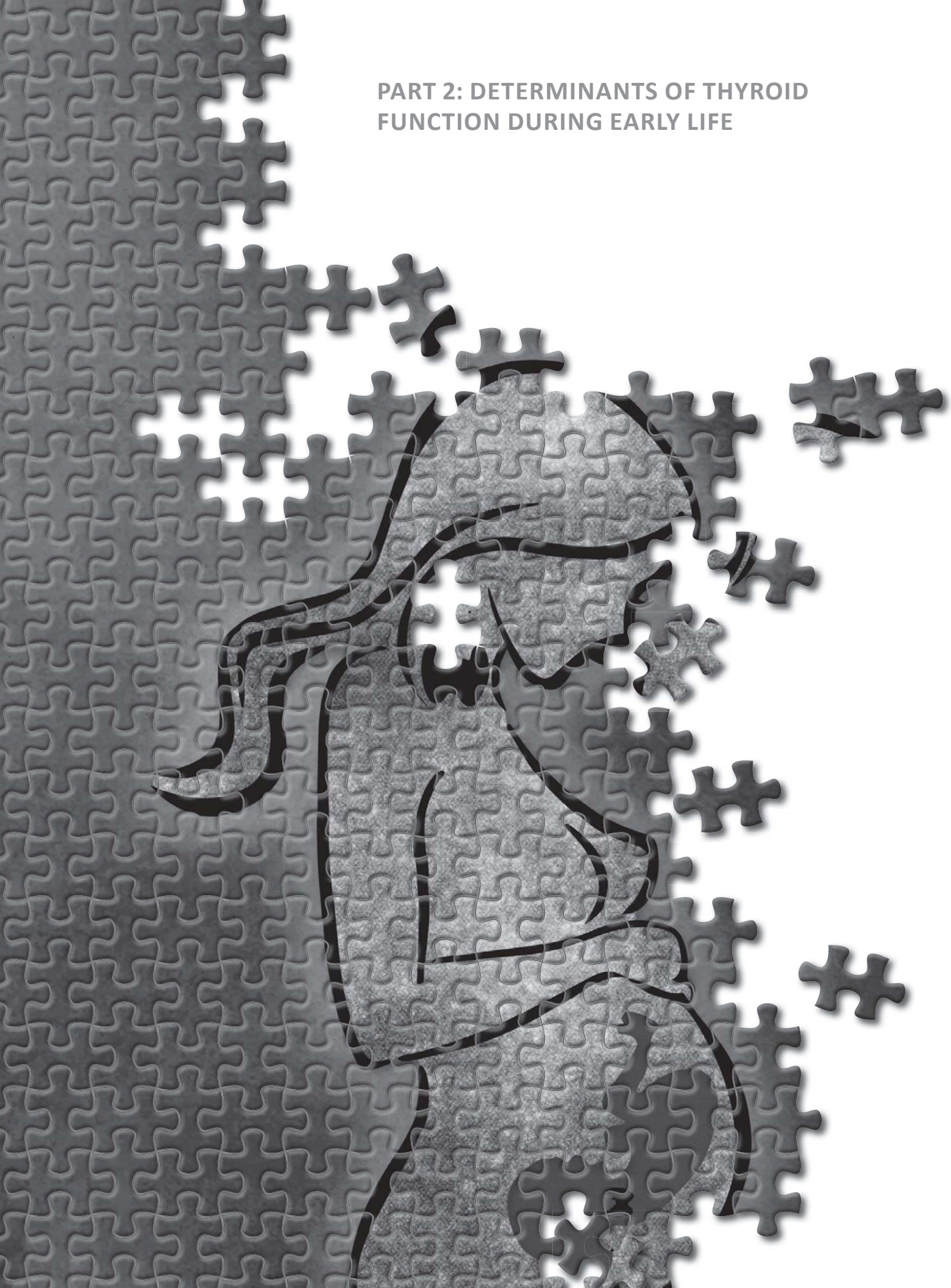
PART 2: DETERMINANTS OF THYROID FUNCTION DURING EARLY LIFE



CHAPTER 7

MATERNAL AND BIRTH CHARACTERISTICS ARE DETERMINANTS OF OFFSPRING THYROID FUNCTION

Tim I. M. Korevaar, Layal Chaker, Vincent W. V. Jaddoe,
Theo J. Visser, Marco Medici, Robin P. Peeters

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ABSTRACT

BACKGROUND Intrauterine adaptation to the outside environment is an important mechanism via which the fetus increases its chance to thrive after birth. Therefore, various maternal, pregnancy and labor related factors are potential determinants of thyroid function of the offspring. Animal studies suggest that very high maternal thyroid hormone levels during pregnancy can alter the development of the hypothalamic-pituitary-thyroid axis set point of the child. However, to what extent maternal and birth characteristics (including maternal thyroid function, smoking, birth weight) are associated with thyroid function of the offspring is currently unknown.

METHODS We selected 4573 mother-child pairs from a large population-based prospective cohort with data available on maternal gestational TSH and FT4 levels and newborn TSH and FT4 (N=3339; at birth) or childhood TSH and FT4 (N=2523; median age six years). We used multivariable (non)linear regression models to study the association of potential determinants (including maternal TSH, FT4, TPOAbs, iodine excretion, age, BMI, smoking status, parity, preeclampsia, fetal distress, gestational age at birth, birth weight, mode of delivery and thyroid function associated single nucleotide polymorphisms (SNPs)) with newborn and childhood TSH and FT4.

RESULTS There was a strong association of maternal TSH and FT4 levels during pregnancy with newborn and childhood TSH and FT4 levels, respectively (both $P<0.0001$). Maternal FT4 was also associated with newborn TSH levels ($P=0.0009$). Birth weight, fetal distress, gestational age at birth and maternal parity were all associated with newborn TSH and/or FT4 ($P<0.0001$) but these associations did not persist into childhood. Genetic risk scores for TSH and FT4 were strongly associated with newborn and childhood thyroid function ($P\leq0.0005$). The association between maternal and offspring thyroid function did not change after correction for genetic risk scores.

CONCLUSIONS In this study, childhood thyroid function was predominantly determined by maternal TSH or FT4 levels and thyroid specific SNPs. Effects of stress related changes in thyroid function at birth were transient. Other potential factors were not associated with offspring thyroid function.

INTRODUCTION

As a key regulator of metabolism, thyroid hormone (TH) plays an important role in the growth and maturation of many target tissues. In order to meet tissue requirements, the production of TH is regulated via the hypothalamic-pituitary-thyroid (HPT) axis with a TH set point that is specific for each individual. During intrauterine development of the HPT axis, the fetal thyroid gland is not functionally matured and therefore the fetus predominantly depends on the placental transfer of maternal thyroxine.[1,2] Animal as well as human studies have suggested that very high or very low maternal TH levels during development may induce a shift in the HPT axis set point of the offspring.[3-10] We have previously shown that maternal thyroid function is positively associated with offspring thyroid function at birth.[11] However, it remains unknown whether differences within the normal maternal thyroid function spectrum are associated with thyroid function of the offspring later in life and whether other factors, including genetics and birth characteristics, may underlie the association of maternal thyroid function with newborn or childhood thyroid function.

Developmental adaptivity refers to the process by which the fetus is prepared for the environment it is about to enter and this adaptivity plays an essential role in providing the optimal chances of survival and reproductive success for the offspring.[12] Developmental adaptivity can lead to fetal programming of adult disease via for example endocrine changes in insulin, androgen, glucocorticoid and/or IGF-1 levels.[12] As such, also prolonged exposure to slightly higher or lower TH levels may affect maturation, metabolic state and eventually the risk of adverse clinical outcomes via an *in utero* shift in the HPT axis set point. However, it remains unknown to what extent maternal and birth characteristics are associated with childhood thyroid function.

Clinical data on the association of maternal thyroid function and characteristics, birth characteristics, and common genetics variants with offspring thyroid function is sparse while this type of data may increase our knowledge on mechanisms that lead to inter-individual differences in thyroid function. Therefore, we investigated the association of maternal and fetal characteristics with thyroid function in newborn and school-aged children in a large population-based prospective cohort study.

METHODS

Study design and participants

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[13] Maternal TSH or FT4 levels were determined in 6065 mothers, in their offspring TSH and FT4 was measured in cord blood samples at birth (N=3388) and during childhood at a median age of six years (N=4306). Women with twin pregnancies (N=128), pre-existing thyroid disease (N=85), thyroid interfering medication usage (N=4) or women with fertility treatment (N=76) and children with thyroid disease or chronic illness (endocrine, inflammatory, autoimmune, cancer or kidney disease; N=12) or thyroid (interfering) medication usage (levothyroxine or growth hormone; N=7) were excluded.

Determinants and covariates

We selected potential determinants based on current knowledge on maternal, pregnancy or birth characteristics and associations with other outcomes[14], biological plausibility and data availability. These included maternal TSH, FT4, TPOAbs, age, BMI, smoking status, parity, preeclampsia, fetal distress, gestational age at birth, birth weight (standardized to gestational age at birth), mode of

delivery and thyroid function associated SNPs. Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating. Information on maternal age and smoking status, was obtained by questionnaires during pregnancy. Maternal weight and length were measured at intake and used to calculate BMI. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. Information on fertility treatment, delivery, pregnancy outcome, date of birth, birth anthropometrics, and the gender of the child were obtained from community midwives, obstetricians, and hospital registries. Preeclampsia was defined according to international criteria which is described in detail previously[15], this diagnosis was crosschecked by certified doctors. Medical and obstetrical history were assessed by questionnaires and answers were crosschecked by certified medical doctors. Models were adjusted for maternal education level and child ethnicity, BMI and age which were obtained by questionnaires and measurement at visit to our research center (same time as blood sampling), respectively. Maternal education level consisted of three categories: no education finished/primary school/secondary phase one, secondary phase two/higher education phase one and higher education phase two. Child ethnicity was determined by country of origin of the child and/or parents and was defined according to the classification of Statistics Netherlands and categorized according to the major ethnic groups in Rotterdam. In a subset of women, urinary iodine/creatinine ratios were available (overlap N=1095 with newborn and N=502 with childhood thyroid function data availability), details on which have been described previously.[16] Although we have previously described the linear association of maternal thyroid function with cord blood thyroid function, these analyses did not take into account the potential effect of important potential confounders/mediators and therefore we believe that new analyses on this association and also the investigation of maternal and birth characteristics as determinants of cord blood thyroid function measurements will add significantly to the previous analyses.[11]

Data on single nucleotide polymorphisms (SNPs) were obtained with the Illumina 670 K platform and subsequent imputation using Phase 2 of the CEPH HapMap project. These data were only available for a subset of children; for N=3111 children with data on newborn TSH or FT4 and N=1833 children with data on childhood TSH or FT4. We calculated a genetic risk score (GRS) for each child according to SNPs that have been associated with thyroid function in adult populations (17-20). Of the total 67 SNPs that have been identified to be associated with thyroid function (18), we were able to obtain data from 56 SNPs that were either the original SNP or a SNP in high linkage disequilibrium (LD; R2>0.9) with the original SNP. Of these 56 SNPs, the SNPs that were in high in LD with each other (R2>0.3) and the SNPs that had an opposite effect estimate compared to the reported effect estimates in adult GWAS studies, were excluded. This resulted in a final selection of 20 and 6 SNPs for TSH and FT4, respectively, that were used for construction of the GRS. The GRS was calculated by multiplying the reported adult effect size and the copy number of effect alleles for each person (0,1 or 2). Due to the ethnic heterogeneity of our study population we performed a sensitivity analysis for all GRS analyses by excluding non-Caucasian children. Further details on genetic data determination, quality controls and infrastructure used have been described previously.[13]

Cord blood thyroid function is notoriously influenced by stress related factors and therefore the variability according to genetics may be lower as compared to later childhood. We investigated this by assessing the difference in standardized effect size estimates of the association between the GRS and either newborn or childhood TSH or FT4.

Procedures

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks; 95% range 9.8-17.5), cord blood samples were obtained directly after birth (median gestational age at birth 40.1 weeks;

95% range 35.9-42.3) and child serum samples were obtained at time of visiting our research center (median age 6 years; 95% range 5.6-7.9). Plain tubes were centrifuged and serum was stored at -80°C. TSH and FT4 were determined in maternal an cord blood serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/ml. Maternal total human chorionic gonadotropin (hCG) levels (same sample as thyroid function) were analyzed in serum using an Immulite XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA), details of which have been described previously.[17] Child TSH and FT4 levels median age 6 years were determined using an electrochemiluminescence immunoassay on the Cobas e601immunoanayzer (Roche Diagnostics, Germany). The intra- and interassay coefficients of variation were 1.1 – 3.0 % for TSH at a range of 0.4 – 0.04 mU/L and 1.6 – 5.0 % for FT4 at a range of 1.6 -24.1 pmol/L. Details on hCG measurements and characteristics have been described in detail previously.[17]

Statistical analyses

Non-linearity of the association between continuous variables and newborn/childhood TSH or FT4 levels was investigated by ordinary least squares linear regression models with restricted cubic splines. We used multiple linear regression models to investigate the association between other variables and newborn/childhood TSH or FT4. Covariates were selected based on biological confounding plausibility, change in effect estimate of the variable of interest, or the reduction of residual variance of the model. Covariates included child sex, age, BMI, ethnicity, household income and maternal education level. We also added hCG to the model based on the underlying physiology and biological effects similar to TSH but this did not change the results. We accounted for the high number of statistical tests (75 in total) by controlling the false discovery rate (Benjamini & Hochberg) using the fdrtool package.[18,19] This methods allows for tailored identification of the expected proportion of false positive results amongst all rejected null hypotheses. We allowed for a maximum of one expected false positive test result which corresponded with a *P*-value of <0.009 which was thus considered as statistically significant. All analyses were performed using R statistical software v 3.03 (package Hmisc, rms, frdtool) or Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

The final study population comprised of N=4573 mother and child pairs with data on maternal gestational TSH and FT4 levels and newborn TSH or FT4 (N=3339) or childhood TSH or FT4 (N=2523”; Figure 1). As compared to mother-child pairs for which data was available for both newborn and childhood thyroid function, mother-child pairs with data availability for only newborn thyroid function were more likely to have a spontaneous delivery, and the mothers were slightly younger, this was opposite for mother-child pairs with data availability for only childhood thyroid function. There was no difference in maternal TSH and FT4 levels, or TPOAb positivity between the mother-child pairs based on data availability.

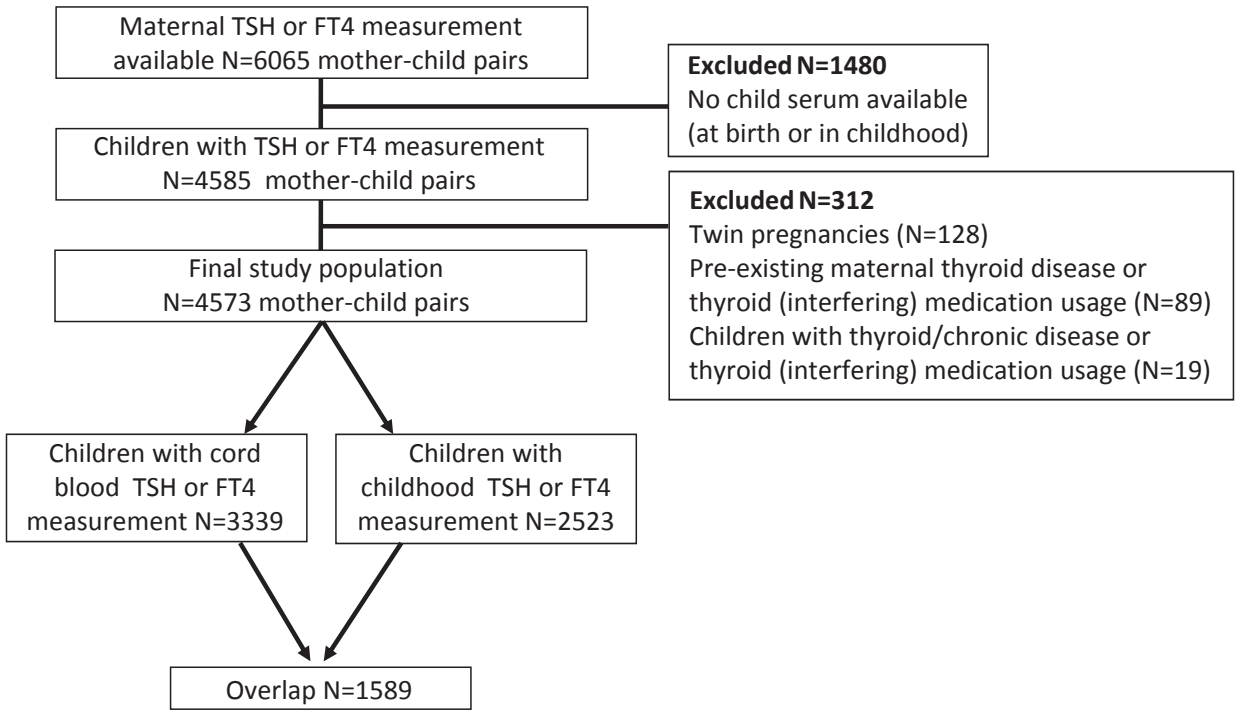
Descriptive statistics of the study population are shown in Supplemental Table 1. The outcomes of standard multiple linear regression models investigating the association between potential fetal programming determinants and newborn thyroid function or childhood thyroid function are shown in Supplemental Tables 2 and 3.

Maternal thyroid function as a determinant of newborn and childhood thyroid function

There was a positive association of maternal TSH with newborn and childhood TSH (Figure 2A-B). Maternal TSH levels explained 1.5% and 4.0% of the variability in newborn and childhood TSH levels, respectively. There was a positive association of maternal FT4 with newborn and childhood FT4 (Figure 2 C-D). Maternal FT4 levels explained 1.6% and 2.9% of the variability in newborn and childhood FT4 levels, respectively.

There was a negative association between maternal FT4 and newborn TSH (Figure 3) and this association remained unchanged after additional adjustment of newborn FT4 (data not shown). Maternal TSH was not associated with newborn or childhood FT4 and maternal FT4 was not associated with childhood TSH (Supplemental Tables 2 and 3; similar when non-linear associations were analyzed). There was no difference in newborn and childhood TSH or FT4 between TPOAb positive and TPOAb negative mothers (Supplemental Tables 2 and 3). All results remained unchanged after addition of a GRS for TSH and FT4 to the model and maternal urinary iodine/creatinine ratio was not associated with newborn or childhood thyroid function (data not shown). There was no effect modification by child gender (data not shown).

FIGURE 1. Flowchart for mother-child pairs in the final study population.



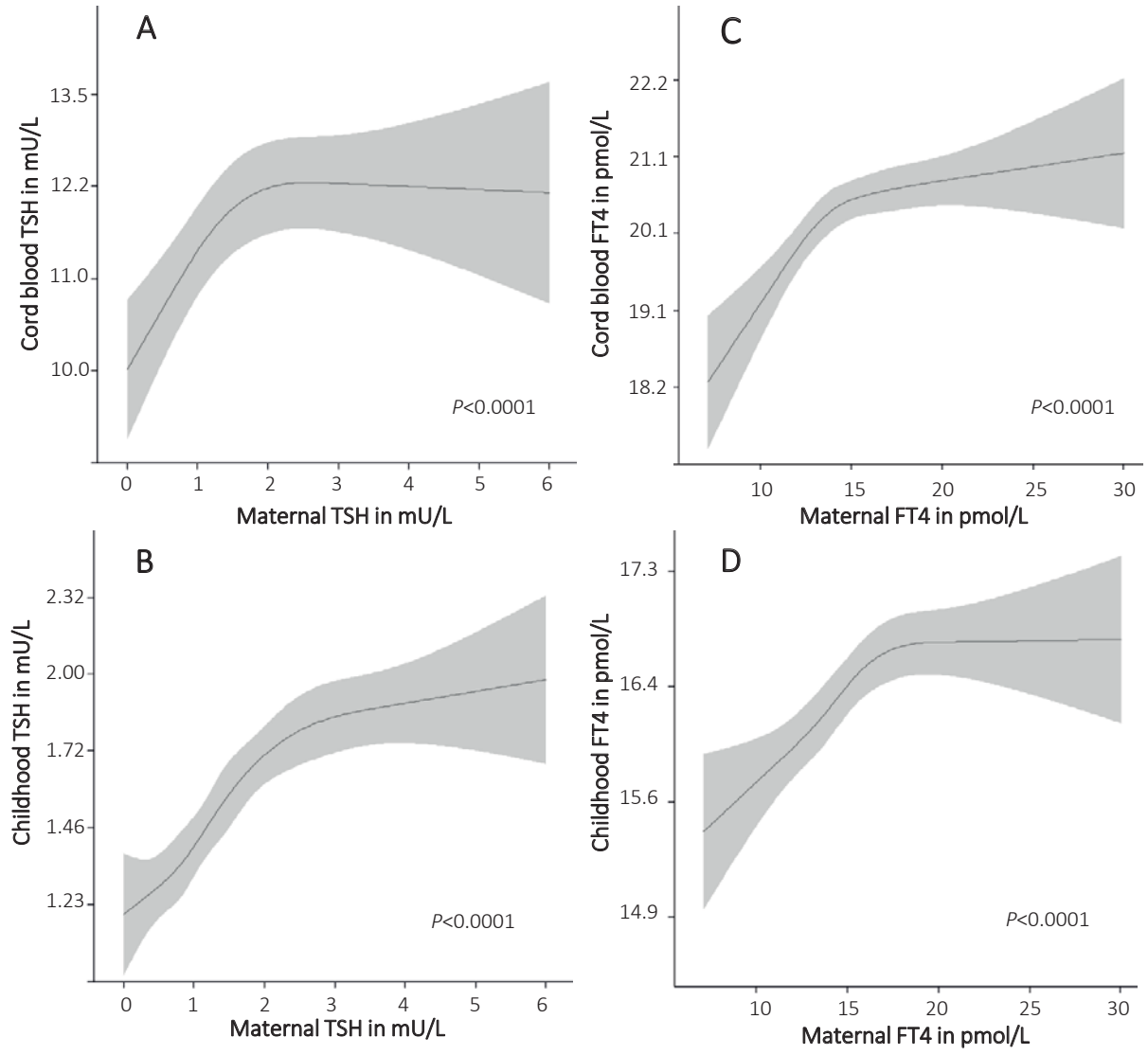
Flow chart showing how the final study population was selected. Differences in selected populations are described in the methods section.

TABLE 1. *The combined effect of genes associated with TSH or FT4 and the association with newborn or childhood thyroid function.*

	newborn TSH			childhood TSH			
	β ±SE	P	Standardized β	β ±SE	P	Standardized β	Difference between newborn vs childhood
Adult GRS TSH	0.218 ±0.43	<0.0001	0.091	0.407 ±0.040	<0.0001	0.230	-60%
	newborn FT4			**childhood FT4**			
	β ±SE	P	Standardized β	β ±SE	P	Standardized β	Difference between newborn vs childhood
Adult GRS FT4	0.055 ±0.019	0.005	0.051	0.097 ±0.017	<0.0001	0.136	-62%

Analyses show beta coefficients with standard error and standardized beta coefficients (for comparison between effect estimates for different outcomes) for the association between a GRS for TSH or FT4 and newborn or childhood TSH or FT4, respectively.

FIGURE 2. The association of maternal TSH and FT4 during pregnancy with thyroid function of the offspring.



Plots show the association between maternal TSH or FT4 levels during pregnancy and child TSH or FT4 at birth or during childhood as predicted mean with 95 percent confidence interval.

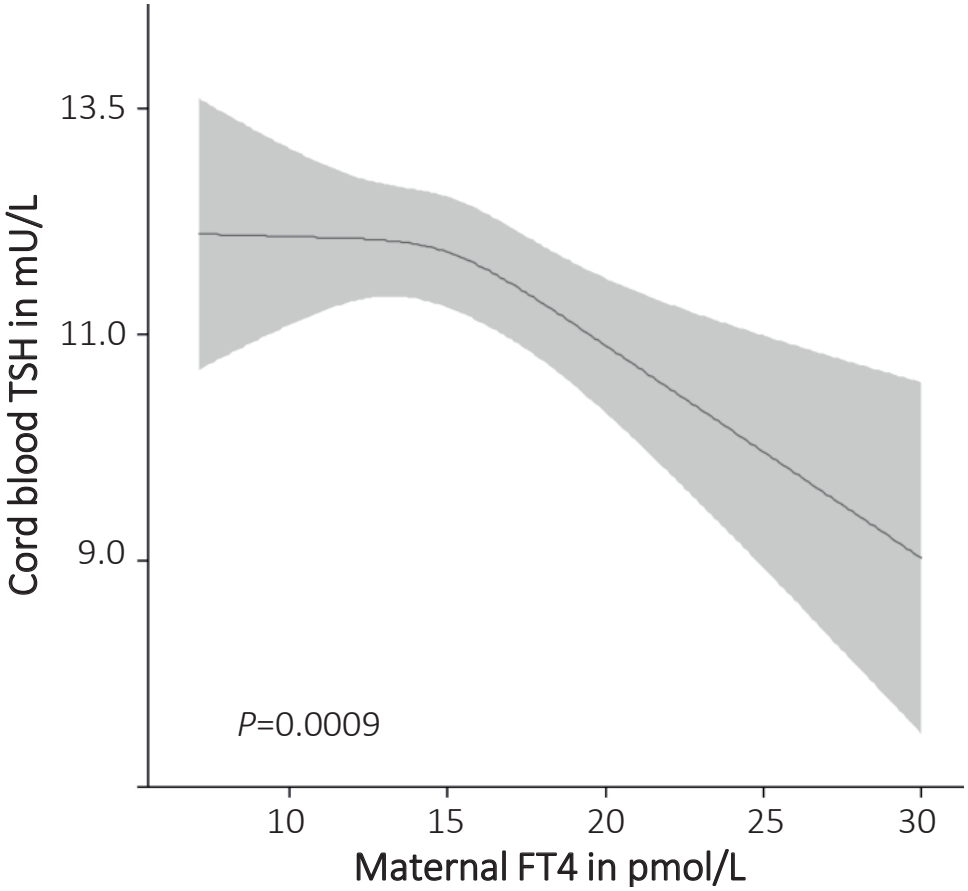
Maternal, newborn and pregnancy determinants of newborn and childhood thyroid function

Newborn TSH was lower in offspring from mothers with higher parity, longer pregnancy duration and in women undergoing cesarean section while newborn TSH was higher in children with fetal distress (Supplemental Table 2). Newborn FT4 was lower in offspring from mothers with a high BMI, lower parity, longer pregnancy duration and when children had a lower birth weight (Supplemental Table 3). There was no association of maternal, newborn or pregnancy factors with childhood TSH or FT4.

Common genetic variants as determinants of newborn and childhood thyroid function

A GRS for TSH was associated with newborn TSH, explaining between 0.8% and 1.0% of the variability in newborn TSH (Figure 4A). A GRS for FT4 was associated with newborn FT4, explaining between 0.2% and 0.3% of the variability in newborn FT4 (Figure 4D). A GRS for TSH was associated with childhood TSH, explaining 5.3% to 5.5% of the variability of childhood TSH (Figure 3B). A GRS for FT4 was associated with childhood FT4 and explained between 1.9% and 3.6% of the variability of childhood FT4 (Figure 3C).

FIGURE 3. The association of maternal FT4 during pregnancy with newborn TSH.



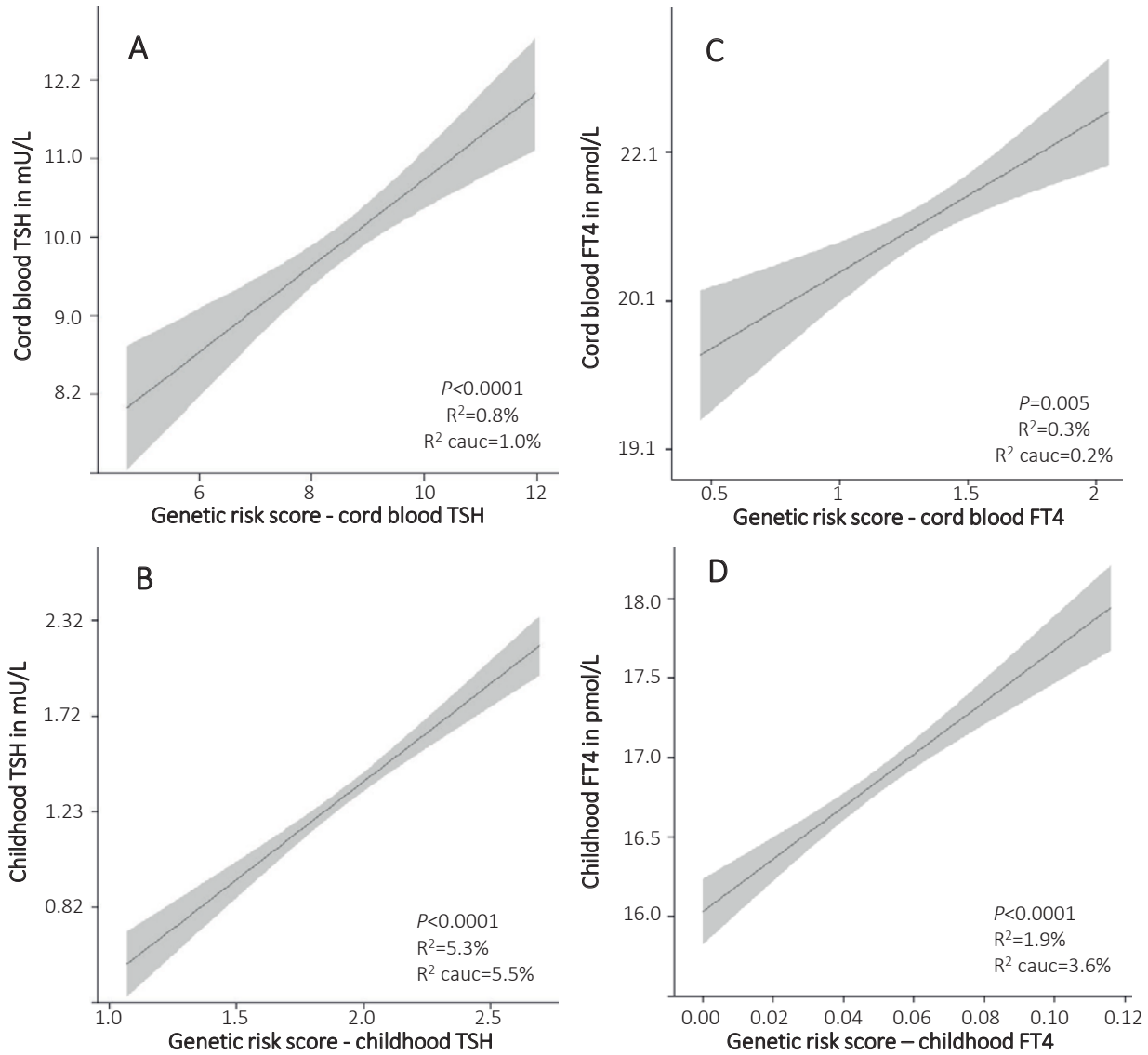
Plots show the association between maternal FT4 levels during pregnancy and child TSH at birth as predicted mean with 95 percent confidence interval.

The combined effect of SNPs on newborn TSH was 60% less as compared to the effects of the same SNPs on childhood TSH (Table 1). The combined effect of SNPs on newborn FT4 was 62% less as compared to the effects of the same SNPs on childhood FT4 (Table 1).

In order to investigate to what extent the effects of the GRS scores and maternal thyroid function overlapped we investigated the explained variability of different models for newborn or childhood thyroid function (Table 2). These analyses showed that the overlap of the explained variability between maternal TSH and a GRS for TSH was 7.8% and 5.5% for newborn and childhood TSH, respectively. The overlap of explained variability between maternal FT4 and a GRS for FT4 was 5.1% for both newborn and childhood FT4.

GWAS data of the mothers was not available, but because mother-child pairs are expected to share about half of their genetics, we investigated whether the GRS scores based on thyroid related SNPs in the offspring would be associated with maternal thyroid function. We found that the offspring GRS for TSH was associated with maternal TSH levels and the offspring GRS for FT4 was associated with maternal FT4 levels (Supplemental Figure 1).

FIGURE 4. The association between genetic risk scores and newborn or child TSH and FT4.



Plots show the association between a genetic risk score for TSH or FT4 and, respectively, TSH or FT4 at birth or during childhood as predicted mean with 95 percent confidence interval.

TABLE 2. *The separate and combined effect of maternal thyroid function and GRS' on newborn or childhood thyroid function.*

	Explained variability			
	Maternal TSH	**GRS for TSH**	**Maternal TSH + GRS for TSH**	**Total overlap**
Newborn TSH	1.51%	0.83%	2.17%	7.8%
Childhood TSH	3.96%	5.30%	8.78%	5.5%
	Explained variability			
	Maternal FT4	**GRS for FT4**	**Maternal FT4 + GRS for FT4**	**Total overlap**
Newborn FT4	1.61%	0.26%	1.78%	5.1%
Childhood FT4	2.86%	1.86%	4.49%	5.1%

Analyses show the r-squared values of linear regression models for either maternal TSH/FT4 alone, GRS for TSH/FT4 alone and both maternal TSH/FT4 plus GRS for TSH/FT4. The total overlap (calculated as (column 3 - (column 1 + column 2)) / column 3).

7

DISCUSSION

In this large population-based prospective cohort study amongst a healthy mother-child pairs we investigated which perinatal maternal and birth characteristics were associated with thyroid function of the offspring at birth and during later childhood. We demonstrated that maternal TSH and FT4 levels are the strongest predictors for both newborn and childhood TSH and FT4 levels, respectively. In addition, maternal FT4 was also associated with newborn TSH levels. Various stress-related factors were associated with newborn TSH and FT4 but these associations did not persist into childhood. We also show evidence that offspring thyroid function may differ according to the inherited SNPs that have been associated with thyroid function.

Intrauterine fetal adaptation to the outside environment is an important mechanism via which the fetus increases its chance to thrive after birth. Animal studies and case reports suggest that offspring exposed to very high maternal thyroid hormone levels have central hypothyroidism, decreased TSH and/or greater resistance to TH at the level of the pituitary.[4-8] Our findings amongst a healthy population demonstrate a strong positive association of maternal TSH and FT4 during pregnancy with offspring TSH and FT4, respectively, suggesting that maternal thyroid function is the strongest determinant of the offspring HPT-axis development in normal physiology. Interestingly, in this study the association of maternal thyroid function during pregnancy with offspring thyroid function attenuated as maternal TSH or FT4 levels increased, suggesting a ceiling effect protecting the offspring from a too extreme HPT-axis set point. Although this is purely speculative, it may also be plausible that the true association between maternal thyroid function during pregnancy and offspring thyroid function has a U-shape (TSH) or inverted U-shape (FT4) since very high levels of FT4 have been shown to cause central congenital hypothyroidism and loss of integrity of thyroid morphology.[3,5,6]

For maternal thyroid function we found that TSH was associated with childhood TSH and maternal FT4 with childhood FT4. There was no association between maternal TSH and childhood FT4 or vice versa. This suggests that there is an important genetic component in the specific establishment of the TSH or FT4 set point. Alternatively, this may suggest that the set point development for TSH and FT4 are not as intertwined as would be expected, but separately determined by factors such as genes and maternal TSH and FT4, respectively. Interestingly, we did find that maternal FT4, which is known to cross the placenta, was associated with TSH in newborns. Although a maximum of 30-50% of newborn T4 levels can be reached via transplacental transportation of maternal T4, this number is likely lower in healthy newborns.[20,21] The association of maternal FT4 is associated with TSH in healthy newborns

remained similar after adjustment for newborn FT4 and a GRS for FT4. Although we cannot exclude the effects of binding proteins, undiscovered genetic variants or other residual confounding, our data confirms that T4 that passes the placenta during late pregnancy influences the newborn HPT axis.

Many research efforts focus on the association of maternal thyroid (dys)function during early pregnancy and child development. It is possible that part of the adverse health outcomes associated with maternal thyroid dysfunction (i.e. cognitive development) are in part mediated by prolonged exposure to slightly higher or lower TH levels. We previously showed that the association of maternal thyroid function during pregnancy with child IQ and MRI outcomes does not change after additional correction for childhood TSH and FT4.[22] However, future research is needed to elucidate to what extent the strong association between gestational thyroid function of the mother and offspring thyroid function can confound the association of maternal thyroid function during pregnancy and other child outcomes.

Cord blood measurements of thyroid hormone have been deemed more unreliable as compared to other serum thyroid function measurements because they are subdue to stress-related factors. The associations of stress-related markers including maternal parity, fetal distress, gestational age at birth, birth weight and mode of delivery, with cord blood TSH and/or FT4 in this study confirm the effects of stress on newborn thyroid function. In addition, we also show that the effect of thyroid SNPs for newborn TSH and FT4 was respectively 60% and 62% lower compared to childhood TSH and FT4 levels. Together with a much lower explained variability of the GRS for cord blood TSH and FT4, these findings reemphasize the role of stress as a determinant of thyroid function in newborns. However, because we were able to study thyroid function at two time points, we demonstrates that the stress related changes in newborn TSH and FT4 are transient and do not persist into early childhood.

We quantified that previously identified SNPs associated with thyroid function in adults explain 5.3-5.5% and 1.9-3.6% of the variability in childhood TSH and FT4, respectively. In a similar approach, a recent study by Taylor et al. amongst a mixed population of children and adults from the UK found that a GRS based on 67 known thyroid function related SNPs explained 7.1% of the variability in TSH but only 1.9% of the variability in FT4 levels.[23] Most likely, the differences between explained variability reported in both studies are due to a difference in GRS methodology since we selected SNPs based effect direction and excluded SNPs in high linkage disequilibrium. This overcomes collinearity and overestimation of allelic effects. In addition, we used adult betas from another population as opposed to betas from our own population. Alternatively, differences between the Dutch and the UK population can also be caused by differences in population characteristics such as iodine status. Interestingly, the study by Taylor et al. also demonstrated that the total genetically explained variability in their mixed childhood/adult population (based on all independent SNPs) is about 24% for TSH and 20% for FT4.[23] The results by Taylor et al. and the quite low explained variabilities reported in this study suggests that the majority of genetic determinants for thyroid function remain to be identified. Potentially, such undiscovered genetic determinants may underlie the association between maternal and offspring thyroid function and/or the low overlap in explained variability of the maternal thyroid function and GRS's of 5.1-7.8%. Future studies are needed to identify more genetic determinants of thyroid function and to investigate to what extent genetic thyroid function determinants overlap between children and adults.

Maternal TPOAb positivity, particularly during pregnancy, has been associated with TPOAb levels in cord blood and an increased risk of TPOAb positivity of the offspring.[10,24,25] In a study by Päkkila et al. amongst 16 year-old offspring, a difference in TPOAb levels and particularly TPOAb positivity between children from TPOAb positive versus TPOAb negative mothers (9.0% versus 3.7% for boys, and 22.7 versus 7.5% for girls; both $P<0.001$) was shown but this difference did not result in differences for TSH or FT4 levels.[10] This is in line with our study, in which we also did not find any differences in TSH or FT4 levels in children from TPOAb positive mothers compared to TPOAb negative mothers.[10] Combined with

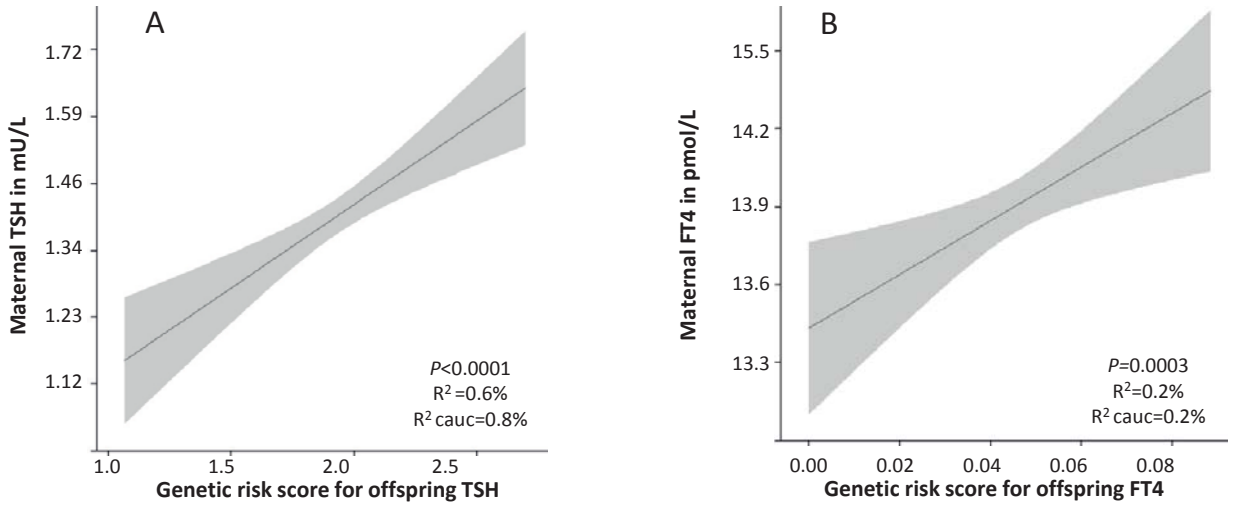
the fact that TPOAbs are in itself biologically inactive, it is likely that maternal TPOAbs have no clinically relevant effects on the thyroid function of the child. Taken together, these data suggest that the clear genetic link of TPOAb positivity does not lead to thyroid function changes before adulthood.

This is the first study to investigate the effects of fetal programming on newborn and childhood thyroid function. We were able to study a large number of mother-child pairs that had extensive pregnancy, phenotype and thyroid function data available which allowed us to test for many potential fetal programming factors. We retained a proper multiple testing correction and used flexible modeling techniques that allowed us to better capture physiological thresholds in the association between maternal thyroid function and offspring thyroid function as compared to other studies. Although the explained variability in child thyroid function by maternal thyroid function was only 1.5-4%, it is important to note that the variation and measurement error of both maternal and childhood thyroid function lead to suboptimal models and underestimation of the effect size. Nonetheless, these number may also suggest that many determinants remain to be identified. Although there were no relevant differences between the groups of data availability (mother, newborn, child overlap), we were limited by the fact that thyroid function data during pregnancy, birth and childhood did not fully overlap. Another potential limitation is the fact that we did not have any data on whether women received thyroid medication after inclusion in the study. Given that all measurements were performed after pregnancy and there is no screening program for thyroid dysfunction during pregnancy it is very unlikely that this would affect our results. We were also limited by the fact that data on child TPOAb levels were unavailable. Nevertheless, in the study by Päkkila et al. TPOAb positivity did not influence child TSH or FT4 levels at age sixteen and it is therefore unlikely that in our younger sample this would have influenced our results.[10] Finally there were no genetic data available from the mothers. This made it impossible to study to what extend the overlap between maternal and child genetics influences the association between maternal and offspring thyroid function.

In conclusion, this study demonstrates that there is a consistent association between maternal thyroid function during pregnancy and thyroid function of her offspring at birth and during childhood. Stress related factors are an important determinant of newborn thyroid function, but these effects do not persist into childhood. More classical markers of fetal programming such as birth weight and maternal smoking were not associated with offspring thyroid function. Future research is needed to investigate whether the strong association between maternal thyroid function during pregnancy and childhood thyroid function could potentially confound or mediate the association of maternal thyroid function during pregnancy and adverse child outcomes.

APPENDIX

SUPPLEMENTAL FIGURE 1. The association of an offspring based genetic risk score with maternal thyroid function.



Plots show the association between maternal TSH and FT4 levels during pregnancy and child IQ as predicted mean with 95 percent confidence interval. Analyses were performed with R statistical package using the RMS package amongst singleton pregnancies after exclusion of women with IVF treatment (N=76) or women with known thyroid disorders or thyroid interfering medication usage (N=89) and were adjusted for gestational age at blood sampling, hCG, maternal age, smoking, BMI, parity, education level, ethnicity, fetal gender and birth weight.

SUPPLEMENTAL TABLE 1. *Descriptive statistics of 4573 mother-child pairs from the Generation R study.*

		Median	(95% range or %)
Gestational TSH	(mU/L)	1.35	(0.04 - 4.51)
Gestational FT4	(pmol/L)	14.8	(10.3 - 22.0)
hCG	(IU/L)	45,193	(12,259 – 106,712)
TPOAb positivity	(N(%))	233	(5.5)
Gestational age at blood sampling	(weeks)	13.2	(9.8 - 17.5)
Maternal age	(years)	30.4	(19.6 – 38.8)
Maternal BMI	(kg/m^2)	23.5	(18.5 - 35.4)
Parity			
0		2449	(53.6)
1		1260	(27.6)
2		398	(8.7)
>2		166	(3.6)
Smoking			
Non-smokers		3159	(69.1)
Stopped smokers		413	(9.0)
Smokers		701	(15.3)
Education level			
High		1926	(42.1)
Middle		1918	(41.9)
Low		429	(9.4)
Preeclampsia		105	(2.3)
Fetal distress		303	(6.6)
Mode of delivery			
Spontaneous*		3604	(78.8)
Caesarean section		399	(8.7)
Breech extraction		132	(2.9)
Other		138	(3.0)
Newborn TSH	(mU/L)	9.42	(3.35 – 33.8)
Newborn FT4	(pmol/L)	20.5	(15.8 – 28.3)
Birth weight	(grams)	3450	(2380 – 4486)
Gestational age at birth	(weeks)	40.1	(36.1-42.3)
Child gender	(boys(%))	2187	(51.2)
Child ethnicity			
Dutch		2444	(53.4)
Moroccan		240	(5.2)
Turkish		304	(6.6)
Surinamese		306	(6.7)
Other Western		405	(8.9)
Other Non-Western		574	(12.6)
Childhood TSH	(mU/L)	2.30	(0.91 - 5.18)
Childhood FT4	(pmol/L)	16.7	(13.7 - 20.6)
Childhood age	(years)	6.0	(5.6-7.9)
Childhood BMI	(kg/m^2)	15.8	(13.7-21.1)

Data are shown after multiple imputation (see methods section).
*Included forceps, expression or vacuum assisted births

SUPPLEMENTAL TABLE 2. *The association of maternal and pregnancy characteristics with offspring TSH.*

	Cord blood TSH			Childhood TSH		
Variable in model	**β**	**±SE**	**P**	**β**	**±SE**	**P**
Maternal TSH[a]	0.039	±0.011	0.0003	0.072	±0.009	<0.0001
Maternal FT4[a]	-0.009	±0.004	0.01	-0.002	±0.003	0.47
Maternal TPOAb+	-0.005	±0.009	0.62*	0.0004	±0.003	0.96*
Maternal Age (per 5 years)	0.021	±0.013	0.10	0.018	±0.011	0.10
Maternal BMI (per 5 points)	0.001	±0.013	0.88	-0.014	±0.012	0.25
Maternal Smoking						
No		ref			ref	
Stopped	-0.016	±0.036	0.67	-0.034	±0.030	0.26
Continued	-0.052	±0.030	0.08	0.002	±0.026	0.82
Parity						
0		ref			ref	
1	-0.133	±0.025	<0.0001	-0.013	±0.021	0.54
2	-0.285	±0.039	<0.0001	-0.064	±0.034	0.06
>2	-0.360	±0.058	<0.0001	-0.011	±0.052	0.83
Preeclampsia	-0.015	±0.076	0.79	0.068	±0.056	0.27
Fetal distress	0.141	±0.044	0.002	-0.061	±0.034	0.08
Gestational age (per week)	-0.032	±0.007	<0.0001	-0.002	±0.005	0.66
Birth weight (per SD[b])	0.028	±0.011	0.11	-0.009	±0.009	0.34
Mode of delivery						
Spontaneous		ref			ref	
Caesarean section	-0.399	±0.042	<0.0001	-0.011	±0.028	0.70
Breech extraction	-0.034	±0.070	0.67	-0.033	±0.047	0.31
Other	-0.050	±0.067	0.55	-0.069	±0.047	0.24

[a] Associations were fit linearly, non-linear associations are shown in Figure 1 and 2.
[b] Birth weight was standardized to gestational age at birth (Niklasson);
* This analysis was performed after removal of maternal TSH from the model due to collinearity.
Table 1 shows the results of a multiple linear regression model for the association between perinatal exposures and offspring TSH at birth and during childhood. All analyses were adjusted for child sex, age, BMI, ethnicity, household income and maternal education level.

SUPPLEMENTAL TABLE 3. *The association of maternal and pregnancy characteristics with offspring FT4 levels.*

	Cord blood FT4			Childhood FT4		
Variable in model	**β**	**±SE**	**P**	**β**	**±SE**	**P**
Maternal TSH[a]	-0.003	±0.003	0.29	0.001	±0.002	0.58
Maternal FT4[a]	0.005	±0.001	<0.0001	0.005	±0.001	<0.0001
Maternal TPOAb+	-0.004	±0.012	0.77	-0.006	±0.010	0.53
Maternal Age	-0.001	±0.003	0.78	-0.004	±0.003	0.12
Maternal BMI	-0.014	±0.003	<0.0001	0.006	±0.003	0.03
Maternal Smoking						
No		ref			ref	
Stopped	-0.005	±0.009	0.56	0.001	±0.007	0.76
Continued	0.020	±0.008	0.01	0.001	±0.006	0.71
Parity*						
0		ref			ref	
1	0.025	±0.006	0.0001	-0.002	±0.005	0.62
2	0.039	±0.010	0.0002	-0.006	±0.008	0.48
>2	0.036	±0.015	0.02	0.017	±0.012	0.16
Preeclampsia	0.000	±0.020	0.78	0.000	±0.013	0.49
Fetal distress	-0.026	±0.011	0.02	0.008	±0.008	0.36
Gestational age birth	-0.015	±0.002	<0.0001	-0.003	±0.001	0.02
Birth weight[b]	0.026	±0.003	<0.0001	0.002	±0.002	0.35
Mode of delivery						
Spontaneous		ref			ref	
Caesarean section	-0.028	±0.011	0.01	0.002	±0.007	0.80
Breech extraction	0.019	±0.018	0.37	-0.016	±0.011	0.06
Other	0.023	±0.018	0.29	0.010	±0.011	0.27

[a] Associations were fit linearly, non-linear associations are shown in Figure 1 and 2.
[b] Birth weight was standardized to gestational age at birth (Niklasson);
[*] This analysis was performed after removal of maternal TSH from the model due to collinearity.
Table 1 shows the results of a multiple linear regression model for the association between perinatal exposures and offspring TSH at birth and during childhood. All analyses were adjusted for child sex, age, BMI, ethnicity, household income and maternal education level.

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CHAPTER 8

SOLUBLE FLT1 AND PLACENTAL GROWTH FACTOR ARE NOVEL DETERMINANTS OF NEWBORN THYROID (DYS)FUNCTION

Tim I.M. Korevaar, Eric A.P. Steegers, Sarah Schalekamp-Timmermans, S. Ligthart, Yolanda B. de Rijke, W. Edward Visser, Willy Visser, Sabine M.P.F. de Muinck Keizer-Schrama, Albert Hofman, Herbert Hooijkaas, Jacoba J. Bongers-Schokking, Henk Russcher, Henning Tiemeier, Vincent W.V. Jaddoe, Theo J. Visser, Marco Medici and Robin P. Peeters

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ABSTRACT

CONTEXT Adequate thyroid hormone availability during fetal and early life is crucial for normal child growth and development. Fetal growth heavily depends on angiogenesis. Placental Growth Factor (PlGF) is a pro-angiogenic factor sharing high homology with Vascular Endothelial Growth Factor (VEGF) whereas Soluble FMS-Like Tyrosine kinase-1 (sFlt1) is a potent antagonist of VEGF and PlGF signaling. Since the thyroid is a highly vascularized organ, we hypothesized that fetal angiogenic factors influence *in utero* thyrogenesis and impair newborn thyroid function. Therefore, we investigated the association between sFlt1 and PlGF on newborn thyroid function.

DESIGN, SETTING, AND PARTICIPANTS sFlt1, PlGF, TSH and FT4 were determined in cord serum of 3525 newborns from a large prospective cohort study. Analyses were adjusted for relevant maternal and child covariates.

RESULTS sFlt1 levels were positively associated with TSH (β 0.07±0.02 mU/L; $P<0.001$) and inversely with FT4 (β -0.58±0.11; $P<0.001$). PlGF showed a positive association with FT4 (β 0.19±0.02; $P<0.001$). Elevated levels of sFlt1 were associated with a 2.8-fold increased risk of hypothyroxinemia ($P=0.04$). Decreased levels of PlGF were associated with a 6.7-fold increased risk of hypothyroxinemia ($P<0.001$). Within the normal range, a dose-dependent effect of sFlt1 on thyroid dysfunction was observed: high-normal sFlt1 levels were associated with a 17.7-fold increased risk of hypothyroxinemia ($P<0.001$) and a 2.7-fold increased risk of hyperthyrotropinemia ($P=0.01$).

CONCLUSION Fetal angiogenic factors sFlt1 and PlGF are associated with newborn thyroid function. Possible effects are most likely mediated through effects on *in utero* thyrogenesis. Abnormal as well as normal-range fetal sFlt1 and PlGF levels influence the risk of impaired newborn thyroid function, which has been associated with adverse neurodevelopmental effects. These data provide important novel insights into the physiology of thyrogenesis and into the etiology of newborn thyroid (dys)function.

INTRODUCTION

Adequate availability of thyroid hormone (TH) *in utero* and during early life is crucial for child growth and development. Fetal TH production starts early in the second trimester and reflects the maturation of the thyroid gland.[1-4] During late gestation, serum TH in the healthy fetus is almost exclusively self-produced whereas it has been shown that in newborns with thyroid abnormalities, maternal transfer of thyroxine can only compensate up to a maximum of ~30% of normal child TH levels.[5,6] As such, the proper intrauterine development of the fetal thyroid gland is essential for obtaining and maintaining adequate TH levels during late gestation and early life. Fetal thyroid function during this period has been associated with important endpoints such as neurocognitive development.[7-11] Although the thyroid is a highly vascularized organ, no vascular determinants of thyrogenesis and/or newborn thyroid (dys) function have been identified so far.

During pregnancy, placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt1 or soluble VEGF receptor 1) are produced by the placenta and are necessary for adequate vascular endothelial homeostasis. PlGF is a pro-angiogenic factor which shares 53% homology with VEGF[12] whereas sFlt1 is a potent soluble antagonist of VEGF and PlGF signalling. An unbalanced ratio of sFlt1/PlGF with high sFlt1 and/or low PlGF levels has been associated with pregnancy complications such as preeclampsia, intrauterine growth retardation (IUGR) and small for gestational age newborns.[13-17]

Various sorts of evidence suggest that factors involved in angiogenesis may affect thyroid tissue. First of all, VEGF blockade in mice leads to a ~60% reduction of vascular density in the thyroid whereas administration of sFlt1 analogues was shown to reduce thyroid capillary density by 68%.[18,19] Second, VEGF blockage has been shown to almost fully suppress the formation of endothelial fenestrations.[18,19] Consequently, VEGF blockage was associated with a significant decrease in FT4 levels whereas administration of sFlt1 was shown to increase levels of TSH.[18,19] Finally, overt or subclinical hypothyroidism is seen as a side effect in 27-85% of patients who undergo VEGF inhibition as anti-angiogenic cancer therapy.[20]

To date, very little clinical determinants of thyrogenesis are known and the effects of PlGF and sFlt1 on fetal thyroid function have not been investigated. Therefore, we investigated the relation between fetal sFlt1 and PlGF concentrations and thyroid (dys)function in cord serum samples from a large population-based study.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands. In total, 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study (n=8,879 enrolled during pregnancy). The study is designed to identify early environmental and genetic causes, and causal pathways leading to normal and abnormal growth, development and health during fetal life, childhood and adulthood. Main exposures of interest include environmental, endocrine, genetic and epigenetic, lifestyle related, nutritional and socio-demographic determinants. Further details have been described previously.[21]

Population for analyses

In 3782 neonates, cord serum TSH, FT4 levels, sFlt1 and PlGF levels were determined at birth (median 40.1 weeks; 95% range 28.4-43.6).[22,23] Neonates born from mothers with twin pregnancies (N=128),

pre-existing thyroid disease (N=48), thyroid (interfering) medication usage (N=1), on fertility treatment (N=47) or with preeclampsia or HELLP syndrome (N=33) were excluded. This resulted in a final population comprising 3525 neonates who were included in one or more analyses.

Serum measurements

Umbilical venous cord blood was sampled immediately after birth and transported to the regional laboratory for processing and storage at -80 C. Measurements were performed between 2008 and 2010 and all measured factors have been shown to be stable during long-term storage.[24,25] TSH and FT4 were determined in cord blood serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY).[26] The intra- and inter-assay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L, <5.4% for FT4 at a range of 14.3-25.0 pmol/L. For 2354 of these neonates, levels of maternal TSH and FT4 during early pregnancy were available (median 13.5 weeks; range 4.5-17.9). Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when >60 IU/ml.[22]

Cord serum levels of sFlt1 and PlGF were obtained from the same cord serum samples as thyroid measurements and analyzed using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics BV, Hoofddorp, The Netherlands). The between-run coefficients of variation for sFlt1 were 2.8% at 5.5 ng/mL and 2.3% at 34.0 ng/mL. The coefficients of variation for PlGF were 4.7% at 24 pg/mL and 3.8% at 113 pg/mL.[17]

Covariates

Gestational age during pregnancy was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[27] Information on maternal age, smoking status, socio-economic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands.[21,28] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status.[21] Information on fertility treatment, mode of delivery, pregnancy outcome, date of birth, birth anthropometrics, and the sex of the child were obtained from community midwives, obstetricians, and hospital registries.

Statistical analysis

For cord serum TSH and FT4 reference ranges were determined by the 2.5th-97.5th percentiles in term births from the full cohort after exclusion of TPOAb-positive mothers, as previously described.[22] Cord serum FT4, TSH, sFlt1 and PlGF were defined as decreased (<2.5th percentile) or elevated (>97.5th percentile). Due to the lack of reference ranges for sFlt1 and PlGF, sensitivity analyses were performed by which sFlt1 and PlGF were also defined as low (<10th percentile) or high (>90th percentile). sFlt1 or PlGF measurements were only available in two predefined overt hyperthyroid newborns but in none of the predefined overt hypothyroid newborns. Therefore, we could not analyze the effects on overt thyroid dysfunction. To investigate the effects of sFlt1 and/or PlGF on the thyroid axis, we selected newborns with an imbalanced thyroid function that was either low-normal (defined as FT4 in the lowest quartile and TSH in the highest quartile) or high-normal (FT4 in the highest and TSH in the lowest quartile; quartiles were used to obtain sufficient numbers).

To achieve normal distribution, TSH values were transformed by the natural logarithm. As we determined previously, this study population is iodine sufficient.[29] TPOAb positive women were not excluded from analyses as sensitivity analyses showed that maternal TPOAbs (constant or positive yes/

no) were not associated with cord serum FT4, TSH, sFlt1, PlGF. Similarly, because sFlt1 and PlGF were not associated with the weight of the placenta, we did not adjust for placental weight.

For covariates with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used.[30] Five imputed data sets were created and pooled for analyses. Maternal smoking, maternal socio-economic status and maternal ethnicity were added to the model (missing due to non-response in 12.1%, 6.8% and 5.5%, respectively). Furthermore, we added gestational age at birth, cord serum TSH and FT4, sFlt1 and PlGF levels, parity, maternal age, birth weight, fetal gender and maternal FT4, TSH and TPOAb levels to the model as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. The associations between sFlt1, PlGF and TSH or FT4 were analyzed after exclusion of outliers (highest and lowest 2.5th percentiles) by using multivariate linear regression and also by comparing mean TSH or FT4 levels across normal range sFlt1 and PlGF quintiles. Logistic regression analyses were performed to investigate the effects of sFlt or PlGF on thyroid dysfunction. Median values were compared using the Mann-Whitney-U test. All analyses were adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, delivery by caesarean section, birth weight and fetal gender. In order to investigate possible effects of maternal thyroid dysfunction on the placenta or child thyroid function , analyses were additionally adjusted for early pregnancy maternal TSH and T4 levels. All analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

Mendelian randomization

In contrast to a randomized study, a study using cross-sectional data cannot show causality. In order to investigate the direction of the association between angiogenic factors and newborn TSH or FT4 we used a Mendelian randomization approach.[31] With a Mendelian randomization, the random allocation of alleles during gamete formation is used to investigate causality. It is a method for obtaining an unbiased estimate of the effect of a putative causal variable without conducting a traditional randomised trial. In the past, single nucleotide polymorphisms (SNPs) have been associated with TSH and FT4 levels.[32] We calculated a genetic risk score for each newborn according to these validated SNPs by multiplying the number of alleles present (0,1 or 2) with the allele's beta for TSH or FT4 and related this score with sFlt1 and PlGF levels. Unfortunately, we were unable to perform a bidirectional analyses because validated SNPs for either sFlt1 or PlGF levels have not been identified. Details on genetic data determination, quality controls and infrastructure used have been described previously.[21]

RESULTS

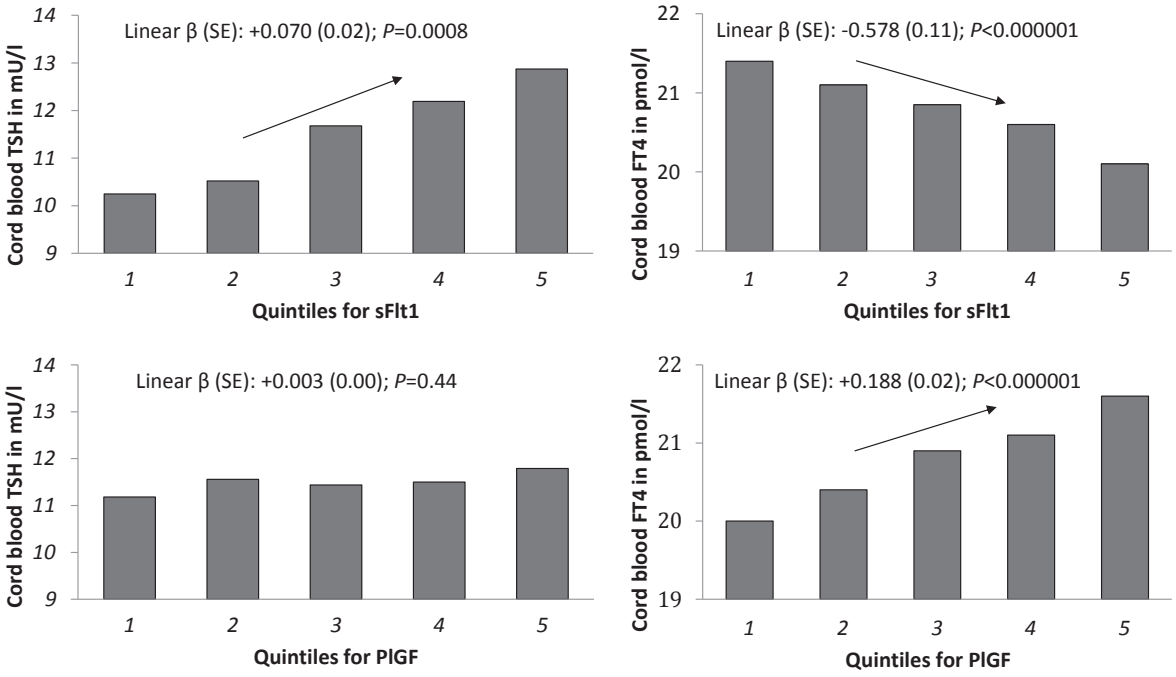
The study population consisted of 3525 neonates, the characteristics of which are shown in Table 1. Cord serum PlGF showed a positive linear association with cord serum sFlt1 (β +0.449; P<0.001) (data not shown). The prevalence of overt thyroid dysfunction was very low (hyperthyroidism in 2 newborns, hypothyroid in none) whereas hyperthyrotropinemia was seen in 82 (2.3%) newborns, decreased TSH in 88 (2.5%), hypothyroxinemia was present in 75 (2.5%) and elevated FT4 in 92 (2.6%).

The relation between cord serum sFlt1, PlGF and TSH or FT4

The relationships of serum sFlt1 and PlGF with TSH and FT4 levels are shown in Figure 1. sFlt1 showed a positive linear association with TSH and a negative linear association with FT4. PlGF showed a positive linear association with FT4 but no association with TSH. Results remained similar after adjustment for covariates and early maternal thyroid function (Supplemental Table 1). Furthermore, gestational age

at birth was an important effect modifier for the association between sFlt1 and FT4 (interaction term sFlt1*gestational age at birth: β -0.25±0.05; *P*<0.001; Supplemental Table 1).

FIGURE 1. *Normal range cord blood sFlt1 and/or PlGF and mean levels of cord blood TSH or FT4.*



Analyses adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, birth weight, fetal gender and birth via caesarean section. TSH values were logarithmically transformed; Highest and lowest 2.5th percentiles for sFlt1 or PlGF were excluded.

Abnormal levels of cord serum sFlt1 and/or PlGF and the risk of newborn thyroid dysfunction.
Following the associations observed, we wanted to investigate whether sFlt1 and/or PlGF were associated with a decreased newborn thyroid function. Since overt newborn hypothyroidism did not occur in our cohort, we analyzed whether elevated sFlt1 or decreased PlGF levels are associated with isolated newborn hyperthyrotropinemia or hypothyroxinemia (Table 2). There was no hyperthyrotropinemia present amongst newborns with abnormal levels of sFlt1 and PlGF and high or low levels of sFlt1 and/or PlGF were not associated with an increased risk of hyperthyrotropinemia. Elevated sFlt1 was associated with a 2.8-fold increased risk of newborn hypothyroxinemia. Decreased PlGF was associated with a 6.7-fold increased risk of newborn hypothyroxinemia. Within hypothyroxinemia FT4 levels with and without decreased sFlt1 or PlGF were 13.5 *vs.* 14.6 pmol/L; *P*=0.33 for sFlt1 and for PlGF 14.21 *vs.* 14.6 pmol/L; *P*=0.18. *Vice versa*, Decreased sFlt1 or increased PlGF levels were not associated with decreased TSH levels or elevated FT4 levels (data not shown).

The effects of normal-range sFlt1and PlGF levels on newborn thyroid dysfunction.
Considering the previous results, subsequent investigations focused on the effects of normal range sFlt1 or PlGF levels on the risk of hyperthyrotropinemia and hypothyroxinemia. These analyses are displayed in Table 3. A high-normal level of sFlt1 was associated with a 2.7-fold increased risk of newborn hyperthyrotropinemia and a 17.7-fold increased risk of newborn hypothyroxinemia. Normal-range PlGF levels did not influence the risk of newborn thyroid dysfunction.

TABLE 1. *Descriptive statistics of 3525 newborns.*

			(95% range)
Gestational age at birth[a]		40.1	(26.6-42.3)
Median TSH	(mU/L)	9.14	(3.41-33.80)*
Median FT4	(pmol/L)	20.5	(15.3-28.1)*
Median sFlt1	(ng/mL)	0.45	(0.09-4.64)
Median PlGF	(pg/mL)	8.8	(4.20-21.36)
Child birth weight[b]		3470 (498)	
Child gender[c] (boys %)		1800 (51.1)	
Maternal age[d]		30.0	(19-39)
Maternal parity[c]			
Nullipara		1953	(55.8)
Primipara		1056	(30.2)
Multipara		493	(14.1)
Maternal smoking[c,e]			
Non-smokers		2584	(73.3)
Stopped smokers		292	(8.3)
Smokers		649	(18.4)
Maternal socio-economic status[c,e]			
Low		416	(11.8)
Middle		1651	(46.8)
High		1456	(41.3)
Maternal ethnicity[c,e]			
Dutch		1818	(51.6)
Moroccan		234	(6.6)
Turkish		307	(8.7)
Surinamese		317	(9.0)
Other western		555	(15.7)
Other non-western		294	(8.3)
Birth via caesarean section[c]		203	(5.8)

* Predefined total-population normal ranges (22)
[a] Data shown as median in weeks
[b] Data shown as mean in grams (SD)
[c] Data shown as n (%)
[d] Data shown as median in years
[e] Data shown after imputation of missing data (12.1% for smoking, 6.8% for socio-economic status and 5.5% for ethnicity).

TABLE 2. *Abnormal sFlt1 and/or PlGF as risk factor for newborn hypothyroxinemia.*

	n	(%)	Hypothyroxinemia[a]	P
Elevated sFlt	5/87	(5.7)	2.76 (1.05-7.27)	0.04
Normal range (reference)	69/3392	(2.0)		
Decreased PlGF	10/67	(14.9)	6.73 (3.02-15.0)	<0.001
Normal range (reference)	53/2863	(1.9)		

[a] Defined according to the 2.5th and/or 97.5th percentiles for TSH and/or FT4. Analyses adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, birth weight, fetal gender and birth via caesarean section.
Newborns with abnormal levels of sFlt1 or PlGF did not present hyperthyrotropinemia.

TABLE 3. *Within normal range cord blood sFlt1 and/or PlGF as predictive factors for newborn thyroid dysfunction.*

	n	(%)	Hyperthyrotropinemia[a]	P [a]	n	(%)	Hypothyroxinemia[a]	P [a]
sFlt1								
1st quintile (reference)	9/664	(1.4)			2/665	(0.3)		
2nd quintile	9/654	(1.4)	0.93 (0.37-2.37)	0.88	7/660	(1.1)	3.82 (0.70-8.59)	0.10
3rd quintile	13/655	(2.0)	1.37 (0.57-3.25)	0.48	11/658	(1.7)	4.98 (1.26-26.5)	0.04
4th quintile	22/659	(3.3)	2.27 (1.03-5.04)	0.04	12/661	(1.8)	6.01 (1.32-27.4)	0.03
5th quintile	26/658	(4.0)	2.70 (1.23-5.93)	0.01	36/661	(5.4)	17.7 (4.52-69.3)	<0.001
PlGF	**n**	**(%)**			**n**	**(%)**		
1st quintile	14/564	(2.5)	0.61 (0.28-1.32)	0.21	18/566	(3.2)	1.88 (0.72-4.94)	0.20
2nd quintile	13/524	(2.5)	0.63 (0.29-1.34)	0.23	9/525	(1.7)	1.14 (0.41-3.23)	0.80
3rd quintile	12/549	(2.2)	0.58 (0.27-1.24)	0.16	5/554	(0.9)	0.62 (0.19-2.04)	0.44
4th quintile	12/605	(2.0)	0.57 (0.27-1.22)	0.15	9/609	(1.5)	1.12 (0.41-3.09)	0.83
5th quintile (reference)	18/535	(3.4)			7/537	(1.3)		

[a] Defined according to the 2.5th and/or 97.5th percentiles for TSH and/or FT4. Analyses adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, birth weight, fetal gender and birth via caesarean section.

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TABLE 4. *Cord blood sFlt1, PlGF and low normal newborn thyroid function.*

	n	(%)	Low normal fetal thyroid function[a]
High[b] sFlt	31/345	(9.0)	1.49 (0.99-2.25) P=0.06
Normal range (reference)	153/2625	(5.8)	
Low[b] PlGF	34/289	(11.8)	1.91 (1.24-2.95) P=0.003
Normal range (reference)	130/2252	(5.8)	

[a] Defined as low-normal (neonatal FT4 in the lowest quartile and neonatal TSH in the highest quartile; N=212/3495) or high-normal (neonatal FT4 in the highest quartile and neonatal TSH in the lowest quartile; N=167/3495).
Analyses adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, birth weight, fetal gender and birth via caesarean section.
[b] High is defined as the highest 10%, low is defined as the lowest 10%.

High/low cord serum levels of sFlt1 and PlGF and effects on thyroid axis feedback mechanisms

We hypothesized that the effects of sFlt1 and/or PlGF would be altered through *in utero* thyrogenesis and biochemical evidence for primary hypothyroidism/hypofunction would argue in favor of this hypothesis. Because overt hypothyroidism was non-prevalent, a proxy for overt thyroid disease and/or primary hypofunction was investigated; low-normal newborn thyroid function (defined as a TSH level in the highest quartile with a FT4 level in the lowest quartile). We found that both high levels of sFlt1 and low levels of PlGF were associated with an increased risk of low-normal thyroid function, as is shown in Table 4.

Causality

To investigate the causal relationship between sFlt, PlGF and thyroid function we performed a Mendelian randomization by calculating a genetic risk score based on previously validated SNPs for TSH and FT4. As is shown in Supplemental Figure 1, the genetic risk scores for TSH and FT4, which were derived from SNPs identified in adult populations, were associated with cord blood TSH and FT4 levels. There was no association or trend between the genetic risk scores and sFlt1 or PlGF.

DISCUSSION

This is the first population-based study which investigates the relation between sFlt1, PlGF and newborn thyroid (dys)function. We demonstrate that cord serum levels of sFlt1 and PlGF are associated with newborn thyroid function, independent of a wide range of possible interfering factors. We also show that abnormal levels of sFlt1 or PlGF increase the risk of impaired newborn thyroid function and that even within the normal-range, sFlt1 and PlGF have considerable effects on thyroid function and the risk of impaired thyroid function.

The thyroid gland of healthy newborns is the most important source of THs during the perinatal period. As such, determinants of thyroid development or function may have profound effects on thyroid related child outcomes or thyroid disorders. Alterations of newborn thyroid function have been linked to neurocognitive development, fetal growth, postnatal acidosis and thyroid function later in life.[9-11,33-36] Furthermore, factors that determine the development of the thyroid gland may be involved in the pathogenesis of transient hypothyroxinemia of prematurity or congenital hypothyroidism. The possible effects of sFlt1 and PlGF on thyroid-related clinical outcomes warrant further exploration.

Considering the high vascularization of the thyroid gland and its rapid maturation in utero, the associations in cord serum levels of sFlt1 and PlGF with TSH and FT4 most likely reflect the extent of intrauterine thyrogenesis. Not only did these associations persist after exclusion of premature newborns, but stratified analyses showed that the associations were even stronger amongst premature newborns, a group in which a very active process of growth and thyrogenesis can be assumed. This hypothesis is also supported by in vivo research which has shown that VEGF blockage reduces thyroid vascular and capillary density and influences thyroid function.[18,19] Furthermore, a recent large meta-analysis of genome-wide association studies amongst non-pregnant individuals demonstrated that genetic variation in the VEGFA gene is associated with thyroid function.[32] Moreover, drugs that block VEGF mediated pathways such as multi-kinase inhibitor Sunitinib, decrease thyroidal blood flow and cause thyroid dysfunction.[20,37]

Alternatively, the relation between (impaired) newborn thyroid function and sFlt1/PlGF may be mediated by altered placental transfer of maternal T4. The type 3 deiodinase (D3), a TH degrading enzyme, is highly expressed in the placenta. In vitro and animal studies suggest that hypoxia not only increases D3, but also results in increased sFlt1 and decreased PlGF levels.[38-42] Apart from disrupting the supply of maternal T4, a similar hypoxia mediated association between D3 and sFlt1/PlGF in fetal tissue could also affect TH.[42-44] Possible other mechanisms by which specifically sFlt1 may exert its effects on TH or thyroid function come from human and animal research focusing on the effects of anti-angiogenic therapy. These mechanisms include the induction of non-placental D3 activity , the inhibition of thyroid peroxidase activity[45] or anti-angiogenic effects on the pituitary.[19] Unlike sFlt1, PlGF induces angiogenesis and its association with increasing levels of FT4 is most likely related to the extent of thyroidal size, development or function at that particular point in time. However, we cannot exclude that VEGF or other angiogenic factors affect intrauterine thyroid development and are also associated with PlGF levels. In turn, this may also explain the different effects of sFlt1 and PlGF on TSH and therefore it seems warranted that future studies also take other (pro)angiogenic factors into account. Another factor that may influence our analyses is arsenic, which has recently been shown to influence sFlt1 gene expression and is a known inhibitor of thyroid function.[46]

Transient hypothyroxinemia is seen in the majority of (very) preterm infants and is characterized by low levels of (F)T4 with normal levels of TSH.[47] It has been associated with neurological deficits such as reduced IQ levels and cerebral palsy.[11] The etiology behind this disease entity remains to be elucidated although various mechanisms have been suggested.[3-5,48,49] We found that the effects of sFlt1 on FT4

were modified by gestational age and that the effects were particularly strong amongst premature newborns. This suggests that especially sFlt1, but perhaps to a lesser extent also PlGF, play a role in the pathophysiology of this hypothyroxinemia phenomenon. Interestingly, high levels of sFlt1 and VEGF have been found in newborn serum and human breast milk and concentrations differ amongst (mothers from) premature and/or intrauterine growth restricted newborns compared to term newborns.[50,51] This might suggest that the influence of angiogenic factors on thyroid development is not restricted to the intrauterine environment.

This is the first study to examine the effects of cord serum sFlt1 and PlGF on cord serum TSH and FT4 and we were able to examine these relations in a large cohort of newborns, with detailed information on possible interfering maternal and/or child factors. We also performed a Mendelian randomization according to previously defined SNPs associated with TSH and FT4 levels. This technique uses the random allocation of alleles during gamete formation is order to investigate causality. This study was limited by the fact that our data was derived cross-sectional and therefore cannot prove causality. However, we identified several strong, specific associations that showed a dose-response relationship and which has biological plausibility. Also, the results of our Mendelian randomization did not refute our hypothesis that sFlt1 and PlGF are determinants of thyroid function in newborns. It must be noted, however, that we cannot excluded the possibility that the latter is caused by a too small sample size. Finally, we were not able to investigate the effects on overt newborn thyroid dysfunction since this is a very sparse outcome. Instead, we investigated isolated abnormal levels of TSH, FT4 and the effects on hypo- and hyperthyroid like thyroid function.

In conclusion, cord serum levels of sFlt1 and PlGF are associated with TSH and FT4 levels in a dose dependent manner. Abnormal levels of sFlt1 and PlGF increase the risk of newborn thyroid dysfunction whereas the risk of thyroid dysfunction was considerably different even within the normal-range of these factors. Our data give insight into newborn thyroid function physiology and suggest that in utero thyrogenesis is influenced by factors which regulate angiogenesis. Further research is warranted to elucidate causality and/or the exact mechanism by which sFlt1 and PlGF influence newborn thyroid development/function and whether these factors play a role in the development of thyroid (related) disease.

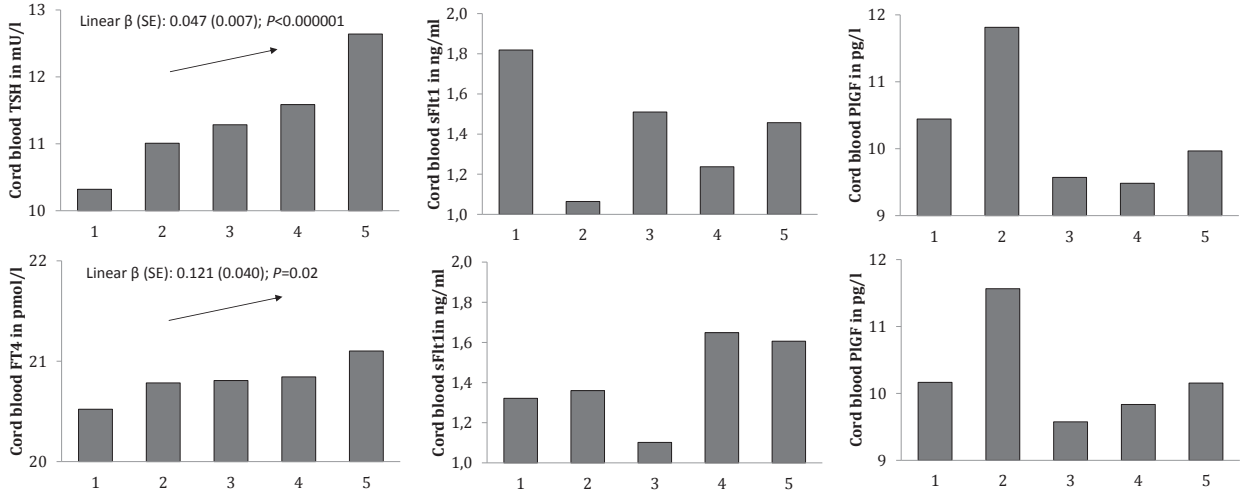
APPENDIX

SUPPLEMENTAL TABLE 1. *Sensitivity analyses for normal range cord blood sFlt1and/or PlGF and cord blood TSH or FT4.*

		Determinants and covariates					
		Linear β (SE)[a]	**P[a]**	**Linear β (SE)[b]**	**P[b]**	**Linear β (SE)[c]**	**P[c]**
sFlt1	TSH	+0.074 (0.02)	<0.01	+0.070 (0.02)	<0.01	+0.089 (0.03)	<0.01
	FT4	-0.749 (0.11)	<0.01	-0.578 (0.11)	<0.01	-0.705 (0.14)	<0.01
PlGF	TSH	-0.001 (0.00)	0.80	+0.003 (0.00)	0.44	-0.002 (0.01)	0.78
	FT4	+0.219 (0.02)	<0.01	+0.188 (0.02)	<0.01	+0.173 (0.03)	<0.01
Effect modification		**Premature delivery[d]**	**P[b]**	**Term delivery[e]**	**P[b]**	**After 40 weeks[f]**	**P[b]**
sFlt1	FT4	-1.737 (0.74)	0.02	-0.340 (0.17)	0.05	-0.660 (0.15)	<0.01

[a] Analyses adjusted for gestational age at birth.
[b] Analyses adjusted for gestational age at birth, maternal age, smoking, SES, maternal ethnicity, parity, birth weight, fetal gender and birth via caesarean section.
[c] Similar to b + child TSH (for FT4 analyses), child FT4 (for TSH analyses), sFlt1 (for PlGF analyses), PlGF (for sFlt1 analyses), maternal T4 and maternal TSH (n=2354).
[d] Defined as gestational age at birth <37 weeks (n=110);
[e] Defined as gestational age at birth 37-40 weeks (n=1421);
[f] Defined as gestational age at birth >40 weeks (n=1994)
TSH values were logarithmically transformed; Highest and lowest 2.5th percentiles for sFlt1 or PlGF were excluded.

SUPPLEMENTAL FIGURE 1. *The associations between a genetic risk score for TSH or FT4 and cord blood levels of sFlt1 or PlGF.*



The genetic risk score was calculated by multiplying the beta per SNP allele associated with TSH or FT4. The SNPs used were selected according to previous findings (32).

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CHAPTER 9

REFERENCE RANGES AND DETERMINANTS OF THYROID FUNCTION IN CHILDHOOD: A SYSTEMATIC REVIEW AND POPULATION-BASED PROSPECTIVE COHORT STUDY

Ibrahim Onsesveren, Mirjana Barjaktarovic, Layal Chaker, Vincent W.V. Jaddoe, Yolanda B. de Rijke, Theo J. Visser, Robin P. Peeters and **Tim I.M. Korevaar**

Submitted

ABSTRACT

BACKGROUND Thyroid dysfunction is associated with suboptimal growth and development in children. In order to clinically define thyroid dysfunction and study its consequences, an adequate definition of reference ranges for TSH and FT4 is crucial. Although various studies have defined reference ranges for TSH and FT4 in pediatric populations, reported cut-offs vary widely and there is no general consensus on how to define an abnormal thyroid function. In addition, little is known about determinants of thyroid function during childhood.

OBJECTIVE To provide an overview of childhood-specific reference ranges for TSH and FT4 by performing a systematic review and to identify clinical determinants of TSH and FT4 in childhood.

METHODS We performed a systematic review on the available studies on childhood TSH and FT4 reference ranges in children. Subsequently, we generated reference ranges by calculating the 2.5th and 97.5th percentile for TSH and FT4 in 4273 children (median age 6.0 years, 95% range 5.7-8.0) from a large population-based prospective cohort. We used (non) linear regression models to study the association of TSH and FT4 with age, sex, anthropometric characteristics, ethnicity, maternal education, time at venipuncture and season.

RESULTS Published cut-offs for TSH and FT4 differed by age range, but considerable differences were also present within age ranges (cut-offs for low TSH: 0.62 to >1 mU/L; high TSH: 2.69 to >10 mU/L; low FT4: 4.5 to >10 pmol/L; high FT4: 5.8 to >10 pmol/L), even with the use of a similar assay. In our Rotterdam cohort, child height, weight, sex, ethnicity were determinants of TSH ($P \leq 0.03$) and FT4 concentrations *(*$P \leq 0.01$), whereas time at venipuncture was a determinant of TSH only. We quantified within our cohort that the variation in a single determinant could lead to a variation of the lower and upper cut-offs between 0.64 to 0.96 mU/L and, 4.30 to 5.62 mU/L for TSH, and13.6 to 14.2 pmol/L and, 20.2-23.0 pmol/L for FT4.

CONCLUSIONS There are considerable differences in the reported reference ranges for child TSH and FT4 across age ranges and different assays, but also within such strata. We identified various determinants of TSH and FT4 concentrations in children. Although these determinants may account for a considerable variation of reference range cut-offs even within a single population, the extent of these differences is relatively small compared to differences between populations and/or assays. Further research is required to reach consensus on reference ranges of thyroid function in children and to define the additive value of age, sex and/or ethnicity-specific reference ranges.

INTRODUCTION

Adequate thyroid function is important for proper growth and development in childhood.[1,2] Hypothyroidism in childhood is associated with cognitive deficits, decelerated growth, delayed skeletal maturation and delayed puberty, whereas overt hyperthyroidism is associated with growth acceleration, advanced bone age and delayed puberty.[2,3] Furthermore, even mild forms of thyroid dysfunction are associated with suboptimal outcomes including weight gain, increased cholesterol levels, anaemia, impaired growth velocity, poor school performance, impaired psychomotor skills and disturbed cognitive development.[4-6]

In order to properly diagnose thyroid disease, adequate reference ranges for TSH and FT4 are essential. The guidelines of the European Thyroid Association for the management of subclinical hypothyroidism in children recommend the use of age-related normative values.[7] However, there is no further consensus on the methodology used to define and interpret these reference ranges for TSH and FT4 during childhood. This complicates the interpretation of thyroid function tests and clinical diagnosis of thyroid dysfunction, as is for example illustrated by the wide range of TSH cut-offs (between 5.5 to 10 mU/L) currently used to define subclinical hypothyroidism during childhood.[7]

Several studies have been performed to define thyroid function reference ranges in paediatric populations.[8-33] Although some of these studies adhere to the recommendations by the International Federation of Clinical Chemistry, there is considerable between-study heterogeneity as these studies have been conducted across various age ranges, using different assays and were performed in populations comprised of different ethnicities and, subdue to different geographical conditions. It is currently unknown to what extent between-study variations in methodology and between-population differences in thyroid function determinants add to this heterogeneity.

Although some studies have indicated that TSH and FT4 reference ranges are influenced by child age, ethnicity, anthropometric characteristics and/or iodine intake, data on determinants of thyroid function during childhood are sparse.[22,34,35] Further knowledge on determinants of TSH and FT4 concentrations during childhood may help to identify specific causes that can underlie an abnormal test result. In addition, such knowledge enables the physician to assess the generalizability of described reference ranges to a specific patient population. Furthermore, knowledge on determinants is important to define mediating and/or confounding factors that can influence studies on the effects of thyroid function on clinical outcomes.

The aim of the current study was to systematically assess and summarize the current literature on thyroid function reference ranges during childhood in order to create a general overview of paediatric TSH an FT4 reference ranges. Subsequently in a large, iodine sufficient paediatric population we aimed to investigate which clinical characteristics are determinants of thyroid function and quantify to what extent these determinants affect reference ranges for TSH and FT4.

METHODS

Systematic review

A systematic literature search of The National Library of Medicine's PubMed database was performed to identify studies published from inception until September 15th, 2016. The following predetermined set of search terms was applied: "((“Thyroid Hormones”[Mesh]) AND (“Reference Values”[Mesh] OR “Reference Standards”[Mesh])) AND “Child”[Mesh]". Two reviewers independently screened the obtained titles and abstracts and subsequently reviewed the full manuscripts for their eligibility for

the systematic review. Finally, the authors cross-checked their results to ensure accuracy. If the authors did not reach complete agreement, the results were discussed with a third reviewer and a consensus opinion was reached. In total, 160 studies were reviewed of which 31 studies were eligible for the systematic review. The reference list of each eligible study was examined and a free text search were performed, resulting in 4 additional relevant studies for the systematic review. Studies using first and second generation assays were excluded to ensure reliability and accuracy in the analyses. The results from the study by Najam et al., were not included in the summary of our results, due to the low number and selection of study subjects.[26]

ORIGINAL STUDY

Design and participants

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands. This study has been described in detail elsewhere.[36] In total, all children with consent for follow-up during childhood (N=8305) were invited to visit the research center of which 6674 children visited. After consent by the mother and child, serum samples were obtained for 4593 children and TSH and/or FT4 were determined in 4286 samples with adequate serum volumes. Children with thyroid disease, chronic illness (endocrine, inflammatory, autoimmune, cancer or kidney disease) or thyroid (interfering) medication usage (levothyroxine or growth hormone) were excluded (N=13).

Determinants and covariates

We selected potential determinants based on the literature, biological plausibility and data availability.[13,22,37] These included age, sex, ethnicity, height, weight, maternal education level (as a marker of social economic status), time and season of venipuncture. Information on these determinants was obtained by questionnaires and measurements during the visit to the research center (on the same day as blood sampling). Medical history was assessed by questionnaires and answers were crosschecked by certified medical doctors. Information on maternal education level was obtained through postal questionnaires. Child ethnicity was determined by the country of origin of the child and/or parents and was defined according to the classification of Statistics Netherlands and categorized according to the major ethnic groups in Rotterdam.[36] These were: Dutch, Turkish, Moroccan, Surinamese, Dutch Antilles, African/Cape Verdian, other Western (European, Oceanian and Caucasian descent Americans /Asians) and other non-Western.

Procedures

Plain tubes were centrifuged and serum was stored at -80°C. Child TSH and FT4 concentrations were determined using an electrochemiluminescence immunoassay on the Cobas e601immunoanalyzer (Roche Diagnostics, Germany). The intra- and interassay coefficients of variation were 1.1 – 3.0 % for TSH at a range of 0.4 – 0.04 mU/l and 1.6 – 5.0 % for FT4 at a range of 1.6 -24.1 pmol/l.

Statistical analyses

Reference ranges for TSH and FT4 in Generation R were defined by the 2.5th and 97.5th percentiles. For analyses aimed to identify thyroid function determinants, TSH concentrations were logtransformed to adhere to model assumptions (results were back-transformed to allow for better interpretation). We used multiple linear regression models to investigate the association between the determinants and

childhood TSH or FT4. Non-linearity of the association between continuous variables and childhood TSH or FT4 concentrations was investigated by ordinary least squares linear regression models with restricted cubic splines utilizing 3-5 knots. As a sensitivity analysis, we repeated the analyses after exclusion of children with TSH or FT4 concentrations outside of the median 95% range to investigate the effect of potential data outliers. We used multiple imputation for potential confounders with missing data. The multiple imputation model included maternal education level, ethnicity of the child, height, weight, age, sex, time of venipuncture and season (missing data in 15.4%, 2.5%, 0.2%, 0.2% and for all other variables 0%, respectively). Five imputed data sets were created and pooled for further analyses. There were no differences between the original and imputed datasets. All analyses were performed using R statistical software version 3.03 (*rms* package) or Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

Systematic review

After exclusions, 26 studies were finally included for extraction of data on TSH and/or FT4 reference ranges (Figure 1A). An overview of all included studies and reference ranges for various age categories is shown in Supplemental Tables 1-8. In general, the variability of reported upper and lower limits reference ranges for TSH and FT4 was highest in the first week of life and became lower as the age of the study population became higher. This was also shown by individual studies that comprised different age categories.[22-28] For studies in children aged ≥1 years, the lower limit for TSH ranged between 0.32 and 1.30 mU/L while the upper limit for TSH ranged from 2.36 to 7.57 mU/L (Table 1). The lower limit for FT4 in these age groups ranged between 8.56 to 18.0 pmol/L, and the upper limit for FT4 ranged between 15.5 to 34.7 pmol/L (Table 1).

FIGURE 1. *Flowchart of the literature search strategy (A) and study population (B)*

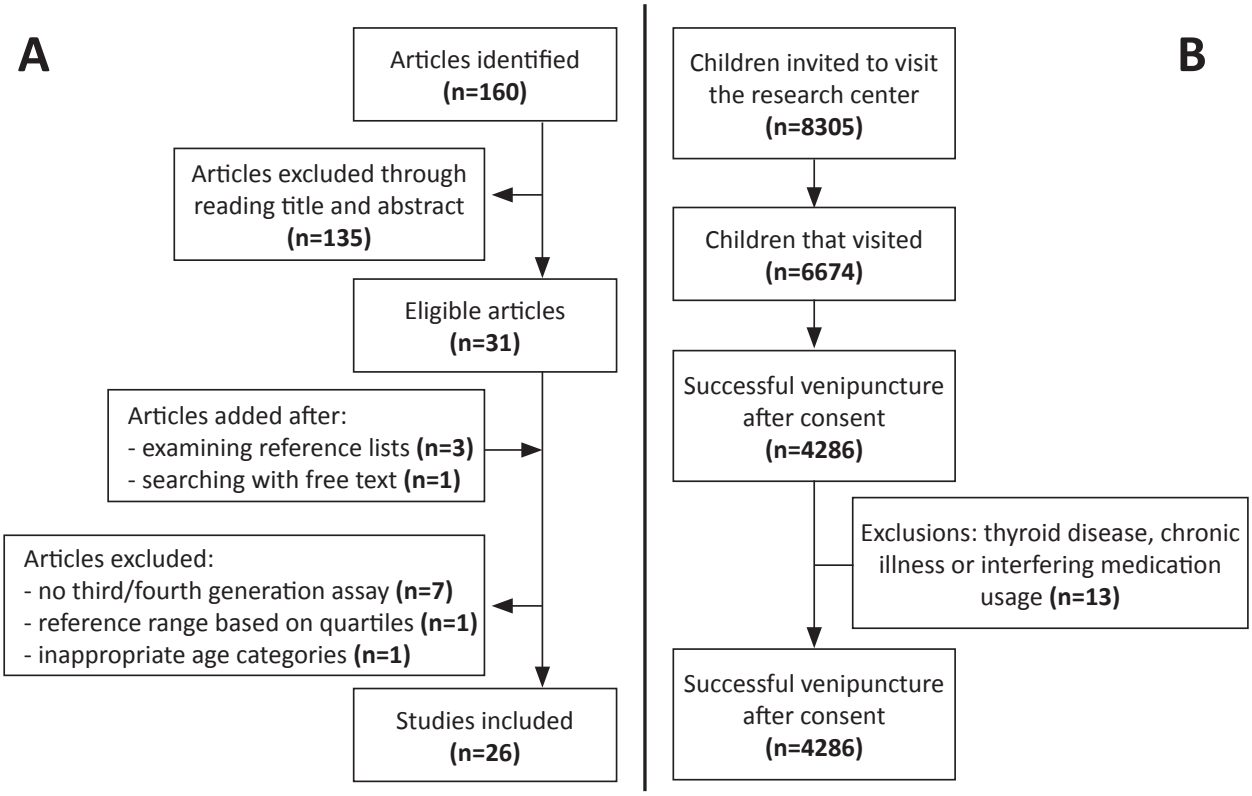


TABLE 1. *The ranges for lower and upper limits of TSH and FT4 reference ranges*

	1 to 7 days	7 days to 3 months	3 months to 1 year	1 year to 5 years	5 years to 10 years	11 years to 20 years
TSH (mU/l)						
Lower limit	0.13 – 1.79	0.16 – 1.80	0.30 – 1.80	0.53 – 1.10	0.48 – 1.30	0.32 – 0.94
Upper limit	9.23 – 57.2	4.38 – 12.56	4.23 – 8.14	3.82 – 7.57	3.36 – 6.51	2.36 – 6.45
FT4 (pmol/l)						
Lower limit	8.9 – 19.9	8.9 – 17.2	9.2 – 12.3	9.4 – 18.0	9.9 – 14.4	8.5 – 14.2
Upper limit	26.8 – 46.6	20.6 – 33.1	19.5 – 25.3	17.0 – 34.7	16.6 – 24.6	15.5 – 25.7

Reference ranges derived from 2.5th and 97.5th percentiles. Reference ranges that were calculated in populations with overlapping age ranges were counted for the category with most overlap.

Original study

Subsequently, we aimed to identify what child characteristics are determinants of thyroid function and to what extent between-population differences in such determinants underlie the large between-study differences in reference range limits for TSH and FT4. Descriptive characteristics of the study population are shown in Supplemental Table 9. After exclusions, the final study population comprised N=4273 children (Figure 1B). There were no considerable differences in characteristics between children with or without data available on TSH or FT4 concentrations (Supplemental Table 10). Child serum samples were obtained at a median age of 6.0 years (95% range 5.7 – 8.0 years) and the majority of subjects were of Dutch origin (57.8%). The number of drawn samples was equally distributed throughout the year with and on average were taken in the afternoon (median time 14:02 hours, 95% range: 11.17-5.17). The median and reference range for TSH concentrations was 2.30 and 0.87 – 5.20 mU/L. The median and reference range for FT4 was 16.8 and 13.8 – 20.8 pmol/L. There was a negative, non-linear association of FT4 with TSH, exhibiting a stable TSH concentration across FT4 concentrations ranging between roughly 12 to 18 pmol/L (Figure 2).

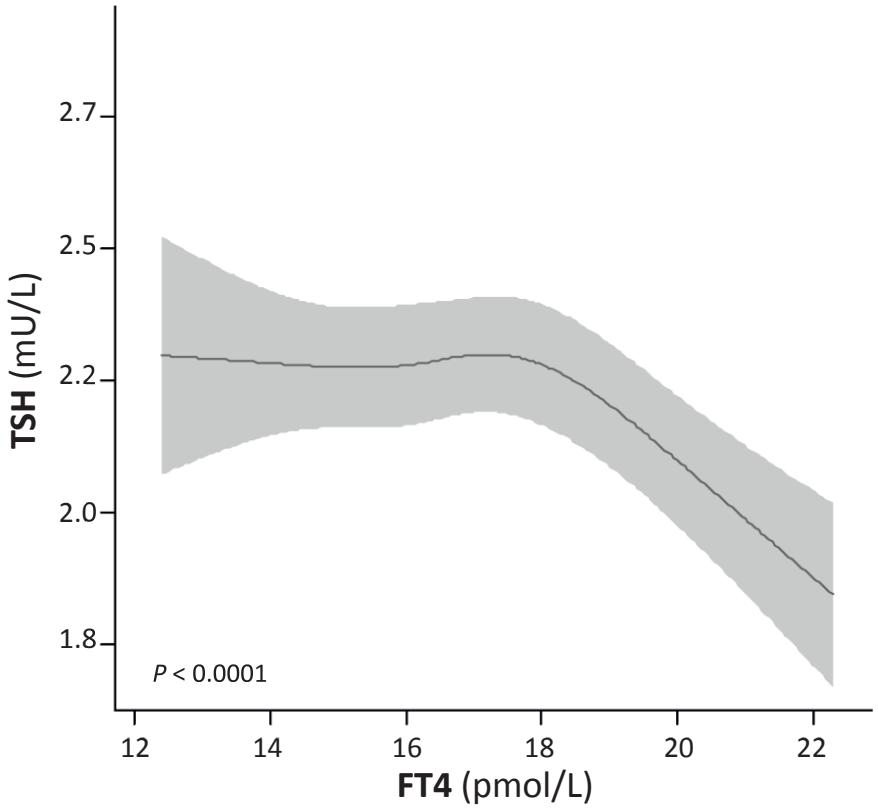
Assessment of thyroid function determinants

Boys had a higher TSH concentration than girls (Figure 3; P<0.0001). TSH differed according to ethnicity, with the lowest concentration in children of Dutch Antilles origin and the highest concentration in Dutch children (Figure 3; P=0.0003). Height was negatively associated with TSH (P=0.0003) and there was a positive linear association of weight with TSH (Figure 3; P=0.03). There was a U-shaped association of time at venipuncture with TSH, indicating that TSH concentrations were higher during the morning and late afternoon (Figure 3; P<0.0001). Age, maternal education or season at venipuncture were not associated with TSH concentrations (Figure 3, Supplemental Figure 1).

Boys had a lower FT4 concentration than girls (Figure 4; P<0.0001). FT4 differed according to ethnicity, with the lowest concentration in Dutch children and the highest concentration in children of non-Western or Surinamese origin (Figure 4; P<0.0001). There was a negative linear association of weight with TSH (Figure 4; P=0.002). There was a non-linear association of age with FT4 concentrations in which FT4 was higher at the lower age range (Figure 4; P=0.01). Season at venipuncture was associated with FT4, with the highest FT4 concentration during autumn and higher maternal education was associated with lower FT4 (Supplemental Figure 1; P=0.006 and P<0.0001, respectively). Height and time at venipuncture were not associated with FT4 (Figure 4). All results remained similar after exclusion of children outside of the reference range for TSH and/or FT4 or when age-standardized values for height or weight were studied.

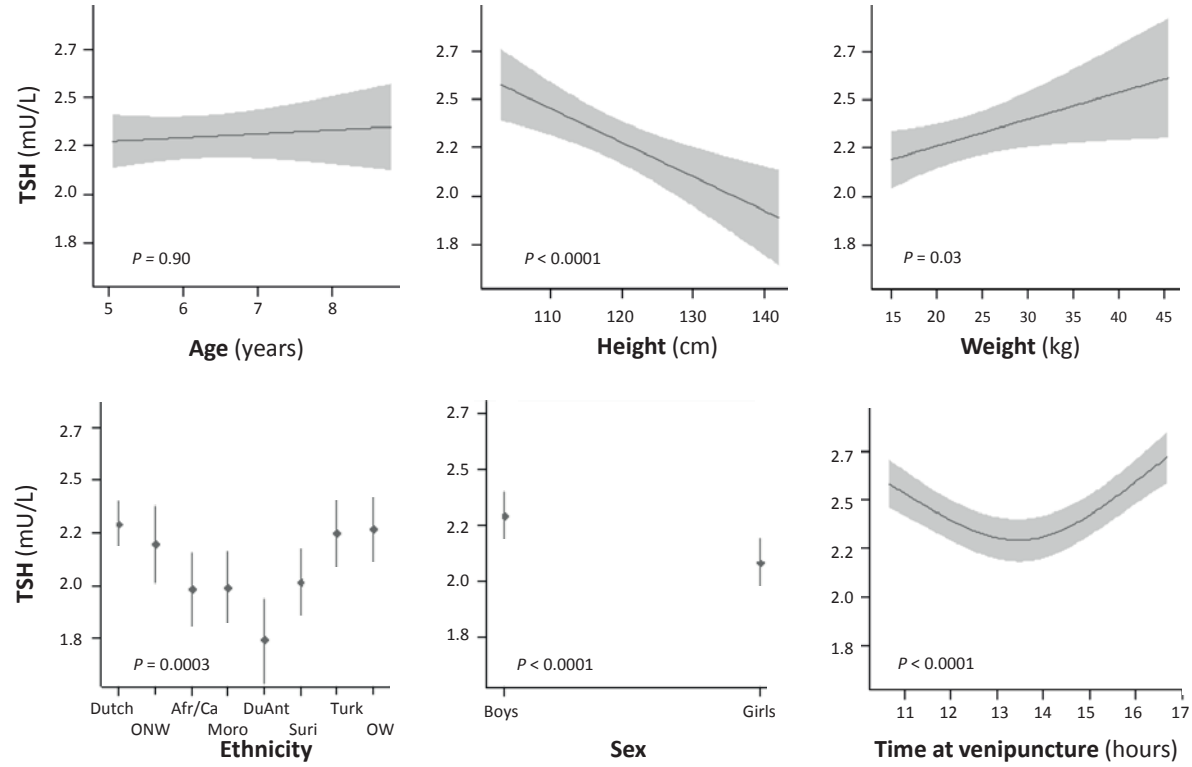
Subsequently, reference ranges were stratified by the studied determinants of thyroid function. The lower limit of TSH in our study ranged between 0.64 to 0.96 mU/L (total population 0.87 mU/L) according to between-individual variation in clinical determinants (Table 2). The upper limit ranged between 4.30 to 5.62 mU/L (total population 5.20 mU/L; Table 2). For FT4, the lower limit ranged between 13.6 to 14.2 pmol/L (total population 13.8 pmol/L) and the upper limit ranged between 20.2 to 23.0 pmol/L (total population 20.8 pmol/L) according to between-individual variation in clinical determinants (Table 2).

FIGURE 2. The association of FT4 concentrations with TSH concentrations.



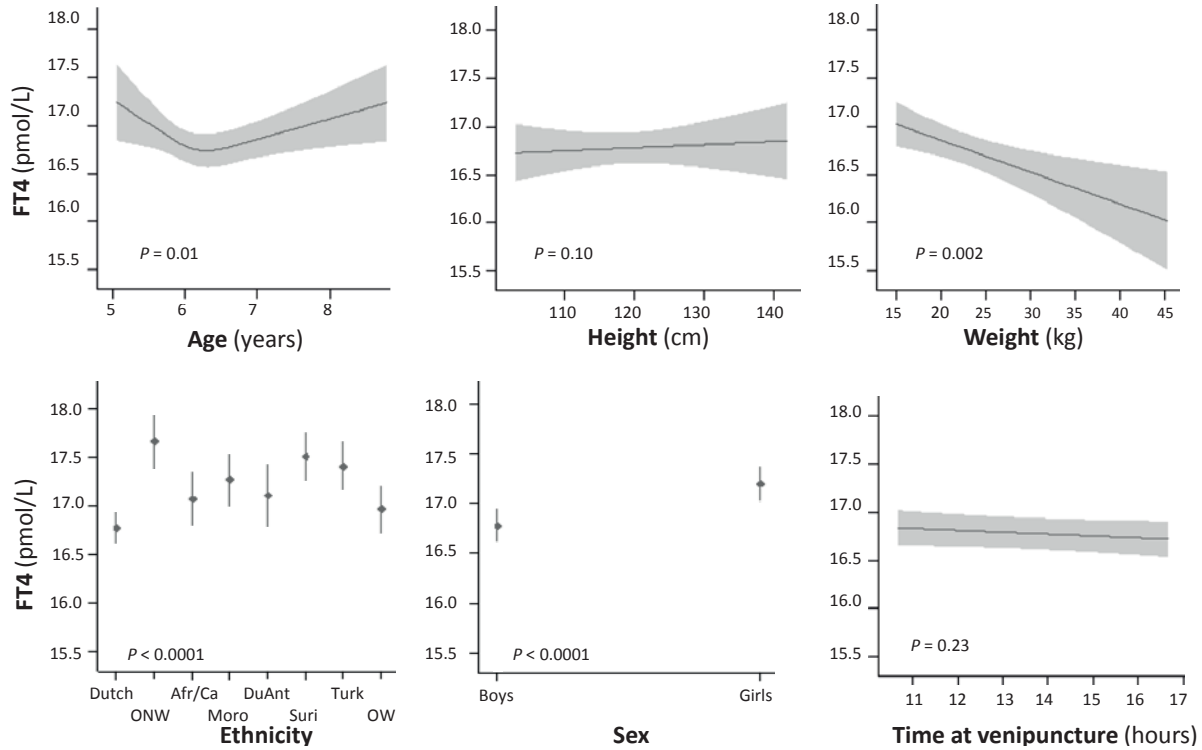
Plot shows the relation between FT4 and TSH in childhood with corresponding 95% confidence interval, adjusted for age, gender, ethnicity, height, weight, time at venipuncture, season and maternal education.

FIGURE 3. The association of potential determinants with TSH concentrations.



Different biological determinants and their relation on TSH with corresponding 95% confidence interval. Every plot has been adjusted for the remaining determinants and further adjusted for season of the year and maternal education status (supplement). OnW: Other non western ethnicities. Afr/Ca: African and Cape Verdian. Moro: Moroccan. DuAnt: Dutch Antilles. Surinam: Surinamese. Turk: Turkish. OthWes: other western ethnicities.

FIGURE 4. The association of potential determinants with FT4 concentrations.



Different biological determinants and their relation on FT4 with corresponding 95% confidence interval. Every plot has been adjusted for the remaining determinants and further adjusted for season of the year and maternal education status (supplement). ONW: Other non-Western ethnicities. Afr/Ca: African and Cape Verdian. Moro: Moroccan. DuAnt: Dutch Antilles. Suri: Surinamese. Turk: Turkish. OW: other western ethnicities

TABLE 2. *Reference ranges for TSH and FT4 stratified by thyroid function determinants.*

	TSH (total population 0.87 – 5.20 mU/L)				FT4 (total population 13.8 – 20.8 pmol/L)			
	Low range	High range	Difference		Low range	High range	Difference	
	(<10%)	(>90%)	Low	High	(<10%)	(>90%)	Low	High
Age	0.88 – 5.28	0.67 – 5.07	0.21	0.21	13.8 – 20.7	13.8 – 21.4	0.0	0.7
Height	0.85 – 5.36	0.79 – 4.97	0.06	0.39	13.7 – 21.4	13.8 – 20.8	0.1	0.6
Weight	0.82 – 5.09	0.86 – 5.19	0.04	0.10	13.7 – 21.1	13.7 – 20.5	0.0	0.6
Time at venipuncture*	0.75 – 4.45	0.89 – 5.38	0.14	0.93	14.0 – 21.2	13.9 – 20.3	0.1	0.9
Gender								
Boys	0.95 – 5.28		0.12	0.18	13.7 – 20.4		0.1	0.7
Girls	0.83 – 5.10				13.8 – 21.1			
Ethnicity								
Dutch	0.90 – 5.35				13.7 – 20.5			
Other non-Western	0.76 – 5.22				13.8 – 21.0			
Cape Verdian/African	0.79 – 5.05				13.9 – 20.5			
Moroccan	0.85 – 4.30		0.32	1.32	14.2 – 20.5		0.6	2.5
Dutch Antillean	0.64 – 4.68				13.7 – 23.0			
Surinamese	0.75 – 5.15				13.7 – 21.7			
Turkish	0.96 – 5.24				14.0 – 21.1			
Other Western	0.95 – 5.62				13.6 – 20.7			
Maternal education level								
Low education	0.87 – 5.17				13.9 – 21.8			
Middle	0.90 – 5.28		0.15	0.16	13.8 – 21.1		0.2	1.6
Higher phase 1	0.85 – 5.12				13.7 – 20.6			
Higher phase 2	1.00 – 5.24				13.7 – 20.2			
Season at venipuncture								
Spring	0.86 – 5.30				13.6 – 20.2			
Summer	0.77 – 4.9		0.19	0.49	13.7 – 21.1		0.3	0.9
Autumn	0.90 – 5.35				13.9 – 20.9			
Winter	0.96 – 5.39				13.7 – 20.7			

** Data shown as median 10% versus highest 10%, lowest 10% values were 0.86 – 5.12 mU/L*

DISCUSSION

Studies that report child reference ranges of TSH and FT4 exhibit considerable heterogeneity with regard to age categories, assay usage and characteristics of study populations.[8-33] In the current study, we provide a literature overview of published reference ranges for thyroid function in children and study which clinical characteristics are determinants of TSH and FT4 and to what extent such differences affect cut-offs for TSH and FT4. We demonstrate large differences in reported reference ranges for TSH and FT4 during childhood that were present across different age-categories, between studies and also between studies utilizing a similar assay. We subsequently identified several thyroid function determinants including child age, sex, ethnicity and anthropometric characteristics using data from a large prospective, population-based cohort from an iodine sufficient area. Already within this population, between-individual variation in a single clinical determinant accounted for variation in the lower and upper cut-offs of between 0.64 to 0.96 mU/L and, 4.30 to 5.62 mU/L for TSH, and 13.6 to 14.2 pmol/L and, 20.2-23.0 pmol/L for FT4.

There is very little consensus on how to define an abnormal thyroid function in children and reference ranges both in a clinical as well as a research setting vary widely.[7] This is at least in part due to the widely differing methodology used to calculate reference ranges for TSH and FT4. For example, some studies define pediatric reference ranges for thyroid function using a non-parametric approach utilizing the 2.5th-97.5th range or the 5th-95th range to define a normal TSH or FT4, while others used a parametric approach defining normality as +/- 1.96 or 2 standard deviations from the mean.[25] Such differences hamper translation of research findings to the clinical setting and also affect the accuracy of a literature summary. The very large differences in pediatric reference ranges for TSH and FT4 as shown in the current study demonstrate the need for standardization of reference range methodology in this field and suggest that further studies are required to optimize clinical diagnosis of thyroid disease in children.

In addition, it is important to note that studies on reference ranges, assessing non-parametric cut-offs, should be performed in a sufficiently sized, non-selected population free of major disease known to affect thyroid function.[38] Many studies lack a sufficiently sized population to generate appropriate reference ranges for different age intervals. Although a minimum of 120 subjects is often proposed for defining reference ranges, this is only recommended as an absolute minimum for the calculation of parametric 90% coverage intervals (e.g. 5th and 95th percentile reference ranges).[39-42] However, because of the high inter-individual variability and skewness for TSH but also to some extent FT4, a minimum of approximately 400 individual measurements per partition is required for these measurements.[39-42] As shown in our literature review, 11 studies presented data derived from less than 100 measurements.[11,15,18,21,23,24,26,28,31-33]

Another important determinant of the large differences in reference ranges for TSH and FT4 is the assay that is used. While most studies used an immunoassay, some studies used equilibrium dialysis and/or LCMS.[8,20] However, even when similar assays were used, there were still large between-study differences. In the study population of 5 to 8-year-old children from the Generation R study, the reference range was 0.87 – 5.20 mU/L for TSH. Studies that used a similar assay report TSH reference ranges that lie anywhere between 0.53 and 6.51 mU/L.[8,12,22] These differences may suggest that also differences in population characteristics may account for some of the between-study differences in thyroid function reference ranges. Although characteristics such as child age and anthropometry have previously been identified as determinants of thyroid function, it is unknown to what extent these may affect reference ranges.[22,34,35] In the current study we show that already within a population from a small geographical area, lower and upper cut-offs for TSH may vary up to 25.4% to 36.7% according to a single thyroid function determinant. For lower or upper FT4 cut-offs, this variation was much lower ranging up to 4.3% and 12.0%. This difference, together with our analysis showing a very stable association between FT4 and TSH, indicating that the hypothalamic-pituitary-thyroid axis is not yet subdue to pathophysiological processes during childhood. Most likely, this is because children in the age range of 5-8 years old are very unlikely to have true thyroidal pathophysiology. Therefore, we speculate that the majority of the between-individual differences in TSH and FT4 presented in this study are cause by differences in the hypothalamic-pituitary-thyroid axis set point, for example based on genetic variation.[43] In order to further clarify the explained variability in thyroid function, genetic studies in children could thus prove to be valuable.

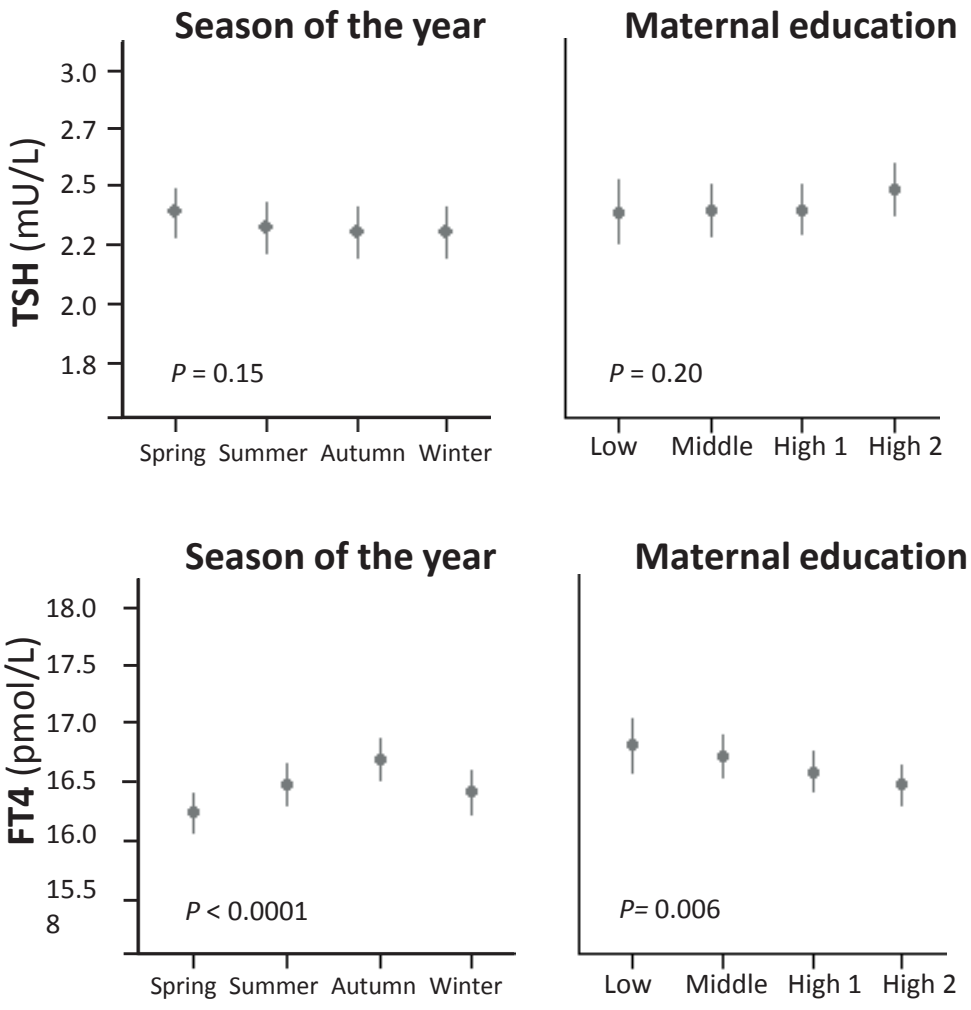
In the current study, we provide a detailed overview of the current literature on thyroid function reference ranges during childhood. Furthermore, we were able to study clinical determinants of thyroid function in a large prospective population-based cohort of children living in an iodine sufficient area. A limitation of this study is the fact that our population comprised a relative narrow age range, therefore, it is not possible to extrapolate our results to other age categories in childhood. Another potential

limitation is that we did not have data available on TPO antibodies. However, it is unlikely that the relative short exposure to thyroid autoimmunity in children already affects thyroid function as is illustrated by the fact that lower FT4 concentrations were not associated with higher TSH concentrations in the current study. Moreover, a Finnish study did not find lower thyroid function in children with TPOAbs.[44] Finally, the observational nature of this study leaves the possibility of residual confounding and the uncertainty about causality within studied associations.

In conclusion, in the current study we demonstrate a large heterogeneity in pediatric thyroid function reference ranges in the current literature. We show that child age, sex, ethnicity, anthropometry and time of venipuncture are determinants of TSH and/or FT4 concentrations and that between-individual variations in these determinants can influence the calculation of reference ranges. The identification of these determinants, and quantification of their effects, can help with the interpretation of thyroid function test.. Future efforts should focus on the generation of evidence based recommendations and consensus to define abnormal thyroid function in children, in order to tackle the large heterogeneity in current studies.

APPENDIX

SUPPLEMENTAL FIGURE 1. The association of potential determinants with FT4 concentrations.



Association of season of the year during blood sampling and maternal education with TSH and FT4 with corresponding 95% confidence interval.

SUPPLEMENTAL TABLE 1. TSH reference ranges of ages 1 day to 1 years.

TSH	Population size	Age					
		1 d – 7 d	8 d – 15 d	15 d – 1 m	1 m – 3m	3 m – 6 m	6 m – 12 m
Abbott Architect							
Bailey et al., 2013	**female 139, male 139**	0.73 – 4.77					
Chaler et al., 2012 (Abbott AxSYM)	**659**		0.92 – 2.30 – 4.38			0.79 – 2.24 – 4.23	0.84 – 2.37 – 4.31
Soldin et al., 2010	**female 152, male 138**				F: 1.12 – 4.47 M: 0.96 – 4.90		
Chan et al., 2009	**combined 71**	0.88 – 5.42					
Access							
Djemli et al., 2004* (Access 2)	**female 12, male 12**	F: 1.5 – 3.3 – 6.5 M: 0.7 – 2.4 – 9.8					
Advia Centaur							
Loh et al., 2015** (Advia Centaur Vitros 5600)	**NC**	0.82 – 12.08	0.91 – 10.63	1.12 – 8.77			
Strich et al., 2012	**425**	1.08 – 11.80			0.68 – 12.56 (to 2 months)	0.62 – 7.3 (from 2 months)	
Kapelari et al., 2008	**64**	0.7 – 3.5 – 18.10			1.12 – 2.85 – 8.21		
Hubner et al., 2002	**460**	0.13 – 9.23 (to 3 days)	0.16 – 8.48 (from 4 days)		0.3 – 5.88 (from 2 months)		
Delfia							
Zurakowski et al., 1999	**female 131, male 158**				F: 0.8 – 6.3 (to 11 months) M: 0.8 – 6.3		
Immulite							
Verburg et al., 2011	**308**	0.32 – 3.11 – 12.27	0.34 – 3.01 – 11.44	0.36 – 2.80 – 9.75	0.32 – 3.25 – 11.21		0.38 – 2.62 – 8.14
Najam et al. 2003	**1104**	1.24 – 8.00 – 27.50 (0-4 days) 0.4 – 5.00 – 13.95 (5-7 days)			0.7 – 9.00 – 17.56 (1 month)		0.31 – 2.5 – 14.51 (1 year)
Elmlinger et al., 2001	**85**	1.79 – 4.63 – 9.69	1.80 – 3.71 – 7.97				
Roche							
Kratzsch et al., 2008 (Roche Elecsys)	**273**	0.71 – 6.88 –57.2	0.99 – 3.89 –10.9			0.61 – 3.42 – 10.7	
Vitros							
Lem et al., 2012† (Vitros Eci Technology)	**512**	1.90 – 5.54 – 17.58 (1 day) 1.40 – 4.64 – 13.10 (2 days) 0.94 – 3.75 – 9.65 (3 days) 0.60 – 2.85 – 6.82 (4 days) 0.58 – 2.14 – 5.58 (7 days)	0.58 – 2.14 – 5.57 (1 month)			0.58 – 2.14 – 5.57 (3 months) 0.58 – 2.14 – 5.56 (6 months)	0.57 – 2.13 – 5.54 (1 year)

**5th and 95th percentile , **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation, NC = size of study population is not clear.*

SUPPLEMENTAL TABLE 2. TSH reference ranges of ages 1-5 years.

TSH	Population size	Age			
		1 – 2 y	2 – 3 y	3 – 4 y	4 – 5 y
Abbott Architect					
Chaler et al., 2012 (Abbott AxSYM)	**1826**	0.97 – 2.46 – 4.35		0.97 – 2.49 – 4.38 (to 6 yrs)	
Soldin et al., 2010	**female 467, male 494**	F: 1.00 – 4.37, M: 0.93 – 4.79	F: 0.85 – 4.07, M: 0.83 – 4.37		
Chan et al., 2009	**female 155, male 152**	F: 0.66 – 4.75, M: 0.67 – 4.50			
Access					
Djemli et al., 2004* (Access 2)	**female 27, male 46**	(from 1 month) F: 1.0 – 2.4 – 5.7 M: 0.7 – 2.1 – 5.9			
Advia Centaur					
Loh et al., 2015** (Advia Centaur Vitros 5600)	**NC**	0.74 – 5.68 (from 2 months)			
Strich et al., 2012	**2782**	0.75 – 6.57			
Kahapola et al., 2012	**combined 215** **female 91, male 124**	0.69 – 1.85 – 3.91 F: 0.65 – 1.94 - 3.82 M: 0.81 – 1.85 – 3.92			
Kapelari et al., 2008	**218**	0.80 – 2.70 – 6.26			
Hubner et al., 2002	**460**	0.42 – 1.98 – 4.79			
Delfia					
Cioffi et al., 2001* (AutoDelfia)	**778**	0.3 – 5.9 (2 years)	1.2 – 5.8 (3 years)	1.0 – 6.1 (4 years)	0.8 – 4.5 (5 years)
Zurakowski et al., 1999	**females 523, males 659**	F: 0.7 – 5.9, M: 0.7 – 6.0			
Immulite					
Verburg et al., 2011	**83**			0.66 – 2.18 – 5.15	
Elmlinger et al., 2001	**<86**	0.63 – 2.04 – 4.12 (from 1 month to 3 years)			0.53 – 1.60 – 2.94 (to 6 years)
Roche					
La'ulu et al., 2016 (Roche E170)	**female 281, male 313**	(from 6months) F: 0.85 – 5.78 M: 1.07 – 7.57		F: 0.80 – 6.90 M: 1.10 – 6.56	
Henderson et al., 2011 (Roche E170)	**45**	(to 6 years) Non-parametric: 1.3 – 5.5 (to 6 years) Robust: 0.7 – 6.1			
Kratzsch et al., 2008 (Roche Elecsys)	**247**	0.60 – 2.60 –5.80		0.63 – 2.57 –5.63	
Vitros					
Lem et al., 2012† (Vitros Eci Technology)	**512**	0.57 – 2.13 – 5.54 (1 year) 0.57 – 2.12 – 5.51 (2 years)			0.56 – 2.08 – 5.41 (5 years)

**5th and 95th percentile, **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation NC = size of study population is not clear*

SUPPLEMENTAL TABLE 3. TSH reference ranges of ages 5-10 years.

TSH	Population size	Age		
		5 – 6 y	7 – 8 y	9 – 10 y
Abbott Architect				
Radicioni et al., 2013	**72**	Pre-pubertal (median age 8.9 y, range: 6.2-12.1 y) 0.87 – 1.95 – 5.19		
Bailey et al., 2013	**female 640 male 640**	0.7 – 4.17 (6mo – <14 yrs)		
Aldrimer et al., 2012	**457**	0.89 – 4.97 (6mo – 12 yrs)		
Chmaler et al., 2012 (Abbott AxSYM)	**1266**		0.82 – 2.36 – 4.74	
Soldin et al., 2010	**female 697 male 537**	F: 0.89 – 4.07 M: 0.81 – 4.07		
Access				
Djemli et al., 2004* (Access 2)	**female 101 male 106**			F: 0.9 – 2.0 – 4.0 M: 1.0 – 1.9 – 3.7
Advia Centaur				
Loh et al., 2015**	**NC**	NA – 5.11 (from 4 years)	0.62 – 4.52	
Strich et al., 2012	**3531**	0.79 – 6.0 (from 6 years)		
Kahapola et al., 2012	**combined 605 female 334 male 271**	0.75 – 1.91 – 3.97 F: 0.79 – 1.90 – 3.95 M: 0.74 – 1.94 – 4.02		
Kapelari et al., 2008	**315**	0.80 – 2.30 – 5.40 (from 6 years)		
Hubner et al., 2002	**460**	0.48 – 1.87 – 4.67 (from 6 years to 10 years)		
Delfia				
Cioffi et al., 2001* (AutoDelfia)	**1368**	0.8 – 4.5 (5 years) 0.9 – 3.9 (6 years)	1.3 – 3.6 (7 years) 1.0 – 4.4 (8 years)	0.7 – 3.8 (9 years) 0.1 – 3.6 (10 years)
Stichel et al., 2000***	**280**	0.54 – 1.69 – 3.36 edian age 10 (3.0; 15.5)		
Zurakowski et al., 1999	**females 562 males 698**	F: 0.6 – 5.1 M: 0.7 – 5.4		
Immulite				
Verburg et al., 2011	**91**		0.80 – 2.35 – 5.24 (7 years)	
Najam et al., 2003	**38**	0.39-19.86		
Elmlinger et al., 2001	**121**		0.80 – 1.86 – 3.48	0.85 – 2.00 – 3.50
Roche				
La'ulu et al., 2016 (Roche E170)	**combined 137 female 252 male 259**	F: 0.85 – 5.83 M: 1.00 – 6.51	1.12 – 5.66 (7 years)	F: 0.94 – 5.40 (from 8 to 9 years) M: 1.14 – 6.41 (from 8 to 9 years)
Iwaku et al., 2013 (Roche ECLIA)	**134**	0.62 - 4.90 (from 4years)	0.53 - 5.16	0.67 - 4.52
Kratzsch et al., 2008 (Roche Elecsys)	**241**	0.76 – 2.38 – 5.35 (from 6 years)		1.04 – 2.54 – 5.61 (to 11 years)
Vitros				
Lem et al., 2012† (Vitros Eci Technology)	**512**	0.56 – 2.08 – 5.41 (5 years) 0.55 – 2.04 – 5.31 (8 years)		

5th and 95th percentile, NA = not available, **18% of samples measured by Vitros 5600, * 3rd – 97th percentile, †reference ranges based on -2 to 2 standard deviation, NC = size of study population is not clear*

SUPPLEMENTAL TABLE 4. TSH reference ranges of ages 11-21 years.

		Age										
TSH	**N**	**11 y**	**12 y**	**13 y**	**14 y**	**15 y**	**16 y**	**17 y**	**18 y**	**19 y**	**20 y**	**21 y**
Abbott Architect												
Ehrenkranz et al., 2015	**52765**	0.53 – 6.45 (1 year to 20 years)										
Radicioni et al., 2013	**368**	Pubertal (range: 9.6 – 17.9 y): 0.76 – 1.75 – 4.51										
Bailey et al., 2013	**518**				0.47 – 3.41							
Aldrimer et al., 2012	**female 119, male 95**			F: 0.43 – 3.35; M: 0.81 – 3.61								
Chaler et al., 2012 (Abbott AxSYM)	**3830**	0.88 – 2.53 – 4.76 (from 9 yrs)			0.88 – 2.28 – 4.65			0.71 – 1.86 – 4.88				
Soldin et al., 2010	**female 1940, male 1293**	F: 0.67 – 3.72; M: 0.79 – 3.98					F: 0.47 – 3.63; M: 0.55 – 3.55					
Chan et al., 2009	**female 201, male 93**	F: 0.47 – 4.13; M: 0.58 – 3.59										
Access												
Djemli et al., 2004* (Access 2)	**female 202, male 200**	F: 0.7 – 1.7 -3.4; M: 0.8 – 1.8 – 3.9				F: 0.6 – 1.5 – 3.7; M: 0.7 – 1.4 – 2.8						
Advia Centaur												
Loh et al., 2015** (Advia Centaur Vitros 5600)	**NC**		0.47 – 3.74									
Strich et al., 2012	**4573**	0.72 – 5.77				0.63 – 6.28						
Kahapola et al., 2012	**F 5121, M 1948**	0.62 – 1.71 – 3.88; F: 0.57 – 1.63 – 3.88; M: 0.84 – 1.82 – 3.79					0.51 – 1.50 – 3.59; F: 0.51 – 1.46 – 3.56; M: 0.52 – 1.63 – 3.72					
Kapelari et al., 2008	**588**	0.70 – 2.10 – 4.61					0.50 – 1.70 – 4.33					
Hubner et al., 2002	**460**	0.53 – 1.78 – 4.58				0.56 – 2.00 – 4.53						
Delfia												
Cioffi et al., 2001* (AutoDelfia)	**1410**	1.0 – 4.4	0.6 – 4.7	0.6 – 4.8	0.9 – 3.0	0.1 – 4.6	0.2 – 3.2					
Zurakowski et al., 1999	**F 1866, M 961**	F: 0.5 – 4.4; M: 0.6 – 4.9					F: 0.5 – 3.9, M: 0.5 – 4.4					
Immulite												
Verburg et al., 2011	**83**		0.66 – 2.11 – 4.88					0.49 – 1.79 – 3.38				
Elmlinger et al., 2001	**419**	0.85 – 3.33	0.86 – 3.21	0.80 – 3.08	0.76 – 2.83	0.70 – 2.55	0.64 – 2.51	0.62 – 2.42	0.52 – 2.36			
Roche												
La'ulu et al., 2016 (Roche E170)	**female 521, male 521**	F: 0.94 – 4.71; M: 0.78 – 6.11	F: 0.88 – 4.71; M: 0.77 – 4.32		F: 0.47 – 4.56; M: 0.65 – 4.16		F: 0.56 – 4.62; M: 0.63 – 4.58					
Iwaku et al., 2013, (Roche ECLIA)	**190**	0.62 - 3.36		0.54-2.78		0.32-3.00						
Henderson et al., 2011 (Roche E170)	**245**	(from 7 years) Non-parametric: 1.0 – 6.2; (from 7 years) Robust: 0.7 – 5.4										
Kratzsch et al., 2008 (Roche Elecsys)	**230**		0.51 – 2.14 – 4.60				0.38 – 1.66 – 3.47					
Vitros												
Lem et al., 2012† (Vitros Eci Technology)	**512**		0.53 – 5.16			0.52 – 5.05			0.51 – 4.93			

*5^{th} and 95^{th} percentile, **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation, NC = size of study population is not clear*

SUPPLEMENTAL TABLE 5. FT4 reference ranges of ages 1 day to 1 years.

FT4	Population size	Age					
		1 d – 7 d	8 d – 15 d	15 d – 1 m	1 m – 3m	3 m – 6 m	6 m – 12 m
Abbott Architect							
Bailey et al., 2013 *	**female 391, male 391**	13.5 – 41.3 (5 to <15 days)		8.8 – 32.6	11.5 – 21.9		
Chaler et al., 2012 (Abbott AxSYM)	**659**		13.0 – 17.2 – 26.8			11.6 – 16.3 – 23.3	11.1 – 16.2 – 24.4
Soldin et al., 2010	**female 298, male 314**	F: 8.9 – 23.9 (to 2 months); M: 10.1 – 23.6				F: 8.9 – 19.2 (from 2 months); M: 9.2 – 20.1	
Chan et al., 2009	**female 43, male 36**	F: 11.0 – 20.6; M: 11.9 – 23.6					
Access							
Djemli et al., 2004* (Access 2)	**female 12, male 12**	F: 11.0 – 13.6 – 22.3; M: 9.8 – 12.2 – 23.2					
Advia Centaur							
Loh et al., 2015** (Centaur Vitros 5600)	**NC**	19.9 – 46.6	17.2 – 33.1	13.2 – 21.8	11.3 – 21.3 (to 2 months)		
Strich et al., 2012	**422**	12.4 – 27.4			12.4 – 21.8 (to 2 months)	10.8 – 19.5 (from 2 months)	
Kapelari et al., 2008	**68**	8.50 – 20.10 – 30.50			9.17 – 15.50 – 25.28		
Hubner et al., 2002	**460**	10.8 – 26.8 (0-3 days)	10.9 – 25.5 (from 4 days)		11.4 – 20.9 (from 2 months)		
Delfia							
Zurakowski et al., 1999	**combined 47**				9.5 – 39.5 (to 11 months)		
Immulite							
Verburg et al., 2011	**308**	8.9 – 18.0 – 33.6	8.9 – 17.9 – 32.9	9.0 – 17.7 – 31.8	10.2 – 17.7 – 25.2		12.0 – 17.4 – 23.1
Najam et al., 2003†	**1109**	8.98 – 17.0 – 26.0 (0 – 4 days) 5.79 – 12.6 – 20.7 (5 – 7 days)			7.1 – 11.4 – 19.1 (1 month)		4 – 10.2 – 18.6 (1 year)
Elmlinger et al., 2001	**<171**	29.6 – 62.4 – 79.2 (nmol/l)	18.0 – 42.3 – 63.6 (nmol/l)			11.1 – 19.7 – 27.3 (to 3 years) (nmol/l)	
LCMS* **							
Soldin et al., 2009 Ultrafiltration at 37°C/25°C	**combined 140**				Combined at 37 °C 1.3 – 2.8 Combined at 25 °C: 0.9 – 1.9 (From 1 month to 1 year)		
Roche							
Kratzsch et al., 2008 (Roche Elecsys)	**258**	10.9 – 17.6 –34.5	12.70 – 17.8 – 30.0			12.30 – 16.9 –23.5	
Vitros							
Lem et al., 2012†† (Eci Technology)	**512**	12.3 – 21.6 – 52.5 (1 week)	12.8 – 21.1 – 44.3 (1 month)			13.4 – 20.3 – 36.8 (3 months) 13.82 – 19.74 – 31.39 (6 months)	13.8 – 19.7 – 31.4 (6 months) 14.1 – 19.2 – 28.2 (1 year)

**5th and 95th percentile, **18% of samples measured by Vitros 5600, †only data on T4, ***Liquid chromatography tandem mass spectrometry, †† reference ranges based on -2 to 2 standard deviation, NC = size of study population is not clear*

SUPPLEMENTAL TABLE 6. FT4 reference ranges of ages 1-5 years.

FT4	Population size	Age			
		1 – 2 y	2 – 3 y	3 – 4 y	4 – 5 y
Abbott Architect					
Chaler et al., 2012 (Abbott AxSYM)	**1826**	11.4 – 16.3 – 24.8		12.0 – 16.6 – 25.0 (to 6 yr)	
Soldin et al., 2010	**female 455, male 476**	F: 9.4 – 18.2; M: 10.6 – 17.0	F: 10.2 – 17.8; M: 10.3 – 17.0		
Chan et al., 2009	**female 93, male 101**	F: 12.0 – 18.6; M: 11.0 – 20.8			
Access					
Djemli et al., 2004* (Access 2)	**female 28, male 47**	F: 9.0 – 11.3 – 16.1 (from 1 month) M: 8.7 – 11.7 – 16.2			
Advia Centaur					
Strich et al., 2012	**2722**	11.7 – 19.0			
Kapelari et al., 2008	**229**	10.5 – 15.7 – 22.4			
Hubner et al., 2002	**460**	11.4 – 14.7 – 19.0			
Delfia					
Cioffi et al., 2001* (AutoDelfia)	**778**	9.9 – 17.3 (2 years)	11.5 – 19.7 (3 years)	8.9 – 22.5 (4 years)	12.2 – 23.6 (5 years)
Zurakowski et al., 1999	**combined 91**	9.0 – 37.2			
ED and LCMS					
La'ulu et al., 2016	**840**	18.0 – 34.7 (from 6 months to 6 years)			
Immulite					
Verburg et al., 2011	**83**			13.4 – 17.7 – 22.1	
Elmlinger et al.,2001	**<51**				12.9 – 17.3 – 23.9 (to 6 years) (nmol/l)
Liquid chromatography tandem mass spectometry					
Soldin et al., 2009 LCMS, Ultrafiltration at 37°C/25C	**combined 274**	Combined at 37 °C; 16.7 – 30.9 Combined at 25 °C; 11.6 – 20.6		(From 3 years to 8 years) Combined/M at 37 °C; 16.7 – 36.6 Combined/M at 25 °C; 11.6 – 20.6	
Roche					
Henderson et al., 2011 (Roche E170)	**46**	Non-parametric: 14.5 – 19.8 Robust: 14.2 – 20.3 (both until 6 years)			
Kratzsch et al., 2008 (Roche Elecsys)	**247**	13.9 – 17.0 –21.4		13.3 – 17.1 –20.3	
Vitros					
Lem et al., 2012† (Vitros Eci Technology)	**512**	14.1 – 19.2 – 28.2 (1 year) 14.3 – 18.8 – 26.3 (2 years)			13.9 – 18.1 – 24.8 (5 years)

Supplemental Table 5. *Reference ranges of fT4 for children from 1 year to 5 years old in literature rounded to 1 decimal place. *5th and 95th percentile, † reference ranges based on -2 to 2 standard deviation*

SUPPLEMENTAL TABLE 7. FT4 reference ranges of ages 5-10 years.

FT4	Population size	Age		
		5 – 6 y	7 – 8 y	9 – 10 y
Abbott Architect				
Radicioni et al., 2013	**72**	Pre-pubertal (median age 8.9 y, range: 6.2-12.1 y) 13.1 – 16.4 – 20.6		
Aldrimer et al., 2012	**471**	10.8 – 16.40 (6 mo to 12 yrs)		
Chmaler et al., 2012 (Abbott AxSYM)	**1266**		11.7 – 16.2 – 24.6	
Soldin et al., 2010	**female 655, male 516**	F: 9.9 – 17.0; M: 10.1 – 16.6		
Chan et al., 2009	**139**	10.9 – 19.0 (from 6 years)		
Access				
Djemli et al., 2004* (Access 2)	**female 103, male 102**			F: 9.6 – 11.6 – 14.5; M: 9.7 – 11.7 – 14.2
Advia Centaur				
Loh et al., 2015 ** (Advia Centaur Vitros 5600)	**NC**		10.91 – 20.58	
Strich et al., 2012	**3452**	11.3 – 18.7 (from 6 years)		
Kapelari., 2008	**327**	10.6 – 15.9 – 20.9 (from 6 years)		
Hubner et al., 2002**	**460**	11.0 – 14.2 – 18.8 (from 6 years to 10 years)		
Delfia				
Cioffi et al., 2001* (AutoDelfia)	**1368**	12.2 – 23.6 (5 years); 5.8 – 29.5 (6 years)	11.3 – 22.8 (7 years); 11.6 – 44.8 (8 years)	11.3 – 18.7 (9 years); 10.8 – 21.8 (10 years)
Stichel et al., 2000***	**280**	6.0 – 9.0 – 13.1 (ug/dL) median age 10 (3.0; 15.5)		
Zurakowski et al., 1999	**combined 57**	8.3 – 34.1		
Immulite				
Verburg et al., 2011	**91**		13.2 – 17.4 – 21.6 (7 years)	
Najam et al., 2003†	**39**	6.6 – 9.3 – 17.5 (5 years)		
Elmlinger et al.,2001	**121**		12.9 – 17.3 – 23.9 (nmol/l)	10.3 – 17.0 – 23.8 (nmol/l)
Liquid chromatography tandem mass spectometry				
Soldin et al., 2009 LCMS, Ultrafiltration at 37°C/25°C	**combined 129**			(From 3 years to 8 years) F/M at 37 °C; 16.7 – 30.9 F/M at 25 °C; 11.6 – 20.6
Roche				
Iwaku et al., 2013 (ECLIA)	**134**	14.4 - 21.5 (from 4 years)	13.8 – 20.7	12.4 – 20.6
Kratzsch et al., 2008 (Roche Elecsys)	**241**	13.7 – 17.0 – 21.7 (from 6 years)		13.2 – 16.9 – 21.6 (to 11 years)
Vitros				
Lem et al., 2012†† (Vitros Eci Technology)	**512**	13.9 – 18.1 – 24.8 (5 years); 13.4 – 17.5 – 24.1 (8 years)		

Table 2 *Reference ranges of FT4 stratified on age (5-10 years) and assay rounded to 1 decimal place. *5th and 95th percentile, **18% of samples measured by Vitros 5600, *** 3rd – 97th percentile, †only data on T4, †† reference ranges based on -2 to 2 standard deviation, NC = size of study population is not clear*

SUPPLEMENTAL TABLE 8. FT4 reference ranges of ages 10-20years.

FT4	Population size	Age									
		11 y	12 y	13 y	14 y	15 y	16 y	17 y	18 y	19 y	20 y
Abbott Architect											
Ehrenkranz et al., 2015	**18344**	10.0 – 18.7 (1 year to 20 years)									
Radicioni et al., 2013	**368**	Pubertal (range: 9.6 – 17.9 y) 10.9 – 13.9 – 19.1									
Bailey et al., 2013	**female 952, male 952**	11.5 – 17.6 (1 year to <19 years)									
Aldrimer et al., 2012	**215**			10.2 – 15.50							
Chaler et al., 2012 (Abbott AxSYM)	**3830**	10.9 – 16.0 – 25.2 (from 9 years)		10.4 – 14.9 – 24.7			9.6 – 14.8 – 25.0				
Soldin et al., 2010	**female 1805, male 1229**	F: 8.5 – 15.7; M: 8.9 – 15.9					F: 8.6 – 15.7; M: 8.6 – 15.7				
Chan et al., 2009	**combined 324**	10.0 – 16.9				10.2 – 17.3					
Access											
Djemli et al., 2004* (Access 2)	**female 204, male 202**	F: 8.8 – 10.7 – 13.5; M: 8.4 – 10.8 – 13.0				F: 8.7 – 10.7 – 13.6; M: 9.5 – 11.8 – 15.0					
Advia Centaur											
Loh et al., 2015** (Vitros 5600)	**NC**	10.2 – 20.1 (from 10 years)									
Strich et al., 2012	**4448**	10.5 – 17.9				10.4 – 18.0					
Kapelari et al., 2008	**597**	10.4 – 15.2 – 21.4				10.6 – 15.2 – 22.6					
Hubner et al., 2002	**460**	10.8 – 13.6 – 18.7				10.7 – 14.4 – 18.7					
Amerlite											
Christofides et al., 1995 (Amerlite – MAB)	**29**	11.8 – 25.2 (age range: 0 – 20 year)									
Delfia											
Cioffi et al., 2001* (AutoDelfia)*	**1410**	10.6 – 20.5	9.4 – 20.7	10.0 – 17.1	9.8 – 16.7	9.8 – 18.9	8.9 – 21.2				
Zurakowski et al., 1999	**combined 158**	7.6 – 31.5					7.0 – 28.7				
ED-LCMS††											
La'ulu et al., 2016	**<1373**	14.2 – 25.7 (from 7 yr)									
Immulite											
Verburg et al., 2011	**83**		12.7 – 21.2						12.3 – 20.9		
Elmlinger et al.,2001	**419**	11.8 – 22.7 (nmol/l)	10.4 – 22.9 (nmol/l)	8.5 – 22.5 (nmol/l)	12.2 – 23.3 (nmol/l)	9.1 – 23.4 (nmol/l)	12.9 – 23.3 (nmol/l)	11.8 – 22.5 (nmol/l)	9.3 – 20.5 (nmol/l)		
LCMS											
Soldin et al*., 2009 Ultrafiltration at 37°C/25C	**female 376, male 256**		F/M at 37C 16.7 – 30.9 F/M at 25C 11.6 – 20.6			F/M at 37C 16.7 – 30.9 F/M at 25C 11.6 – 20.6		F/M at 37C 16.7– 30.9 F/M at 25C 11.6 – 20.6			
Roche											
Iwaku et al., 2013 (Roche ECLIA)	**190**	13.1 – 19.6		12.4 – 19.6		12.2 – 19.7					
Henderson et al., 2011(Roche E170)	**250**	Non-parametric: (from 7 years) 13.0 – 20.3 Robust: 12.3 – 20.0		Non-parametric: 12.8 – 20.6 Robust: 12.1 – 20.2							
Kratzsch et al., 2008 (Roche Elecsys)	**230**		12.0 – 15.4 –22.0				12.2 – 17.0 –22.2				
Vitros											
Lem et al., 2012† (Vitros Eci Technology)	**512**		12.7 – 23.3			12.3 – 22.8			12.0 – 22.3		

**5th and 95th percentile, **18% of samples measured by Vitros 5600, † reference ranges based on -2 to 2 standard deviation, †† Equilibrium dialysis-liquid chromatography tandem mass spectrometry, NC = size of study population is not clear*

SUPPLEMENTAL TABLE 9. Descriptive statistics of study population.

		N (%)	or 95% range
Age	(years)	6.0	(5.7-8.0)
Sex	(boys, %)	2202	(51.5%)
Length	(cm)	119	109-133
Weight	(kg)	22.6	17.6-34.7
Ethnicity child			
Dutch		2402	(57.8)
Moroccan		253	(6.1)
Dutch Antillean		132	(3.2)
Surinamese		296	(7.1)
Turkish		296	(7.1)
Cape Verdian/African		218	(5.2)
Other Western		351	(8.4)
Other non-Western		211	(5.1)
Maternal education level			
No education finished/low		457	(12.6)
Middle		1110	(30.5)
Higher phase 1		1002	(27.5)
Higher phase 2		1072	(29.4)
Season			
Spring		1159	(27.1)
Summer		1074	(25.1)
Autumn		1111	(26.0)
Winter		929	(21.7)
Average time of venipuncture		14:02	(11:17-5:17

SUPPLEMENTAL TABLE 10. Differences in characteristics according to missingness of thyroid function.

	Age (years)		Height (cm)		Weight (kg)		Season		Maternal education level	
	Beta (β)	**P-value**	**Beta (β)**	**P-value**	**Beta (β)**	**P-value**	**Beta (β)**	**P-value**	**Beta (β)**	**P-value**
Independent										
Missing TSH	-0.057	*P*<0.001	-0.864	*P*<0.001	-0.369	*P*=0.001	0.050	*P*>0.05	-0.081	*P*>0.05
Missing FT4	-0.056	*P*<0.001	-0.838	*P*<0.001	-0.351	*P*=0.001	0.053	*P*>0.05	-0.082	*P*>0.05

Non-response analysis assessed by linear regression analyses with the clinical characteristics as the dependent variable and missing TSH/FT4 independent variables.

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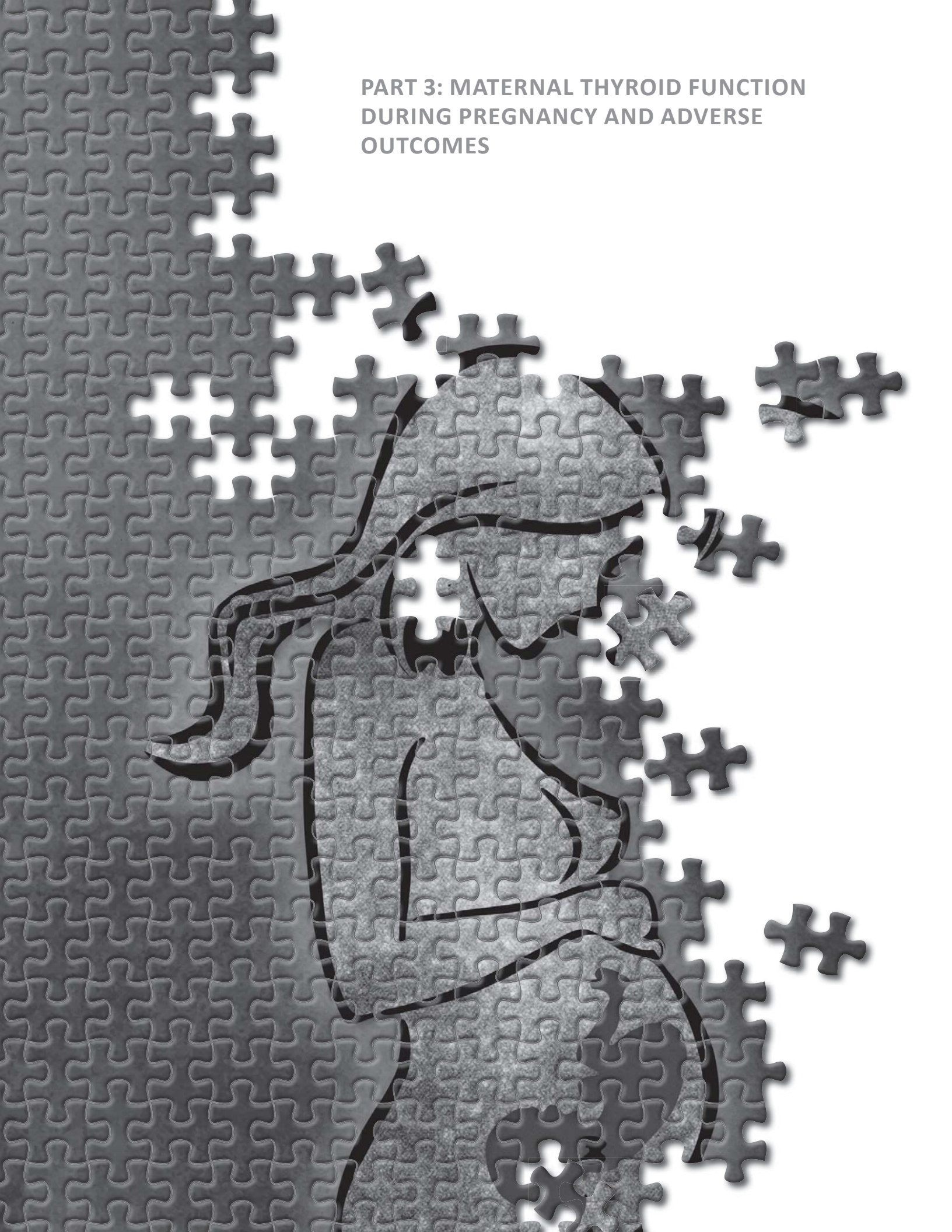
PART 3: MATERNAL THYROID FUNCTION DURING PREGNANCY AND ADVERSE OUTCOMES



CHAPTER 10

MATERNAL EARLY-PREGNANCY THYROID FUNCTION IS ASSOCIATED WITH SUBSEQUENT HYPERTENSIVE DISORDERS OF PREGNANCY

Marco Medici*, **Tim I.M. Korevaar***, Sarah Schalekamp-Timmermans, Romy Gaillard, Yolanda B. de Rijke, W. Edward Visser, Willy Visser, Sabine M.P.F. de Muinck Keizer-Schrama, Albert Hofman, Herbert Hooijkaas, Jacoba J. Bongers-Schokking, Henning Tiemeier, Vincent W.V. Jaddoe, Theo J. Visser, Robin P. Peeters and Eric A.P. Steegers

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ABSTRACT

CONTEXT Hypertensive disorders during pregnancy are associated with a wide range of maternal and fetal complications, and only few risk factors are known for the development of these disorders during pregnancy. Conflicting and limited data are available on the relation between thyroid (dys)function and the risk of hypertensive disorders of pregnancy.

OBJECTIVE To study the associations between early-pregnancy thyroid dysfunction, thyroid function within the normal range, and the risk of hypertensive disorders.

DESIGN, SETTING AND PARTICIPANTS In early pregnancy, serum TSH, FT4 and TPO-antibody (TPOAb) levels were determined in 5153 pregnant women. No interventions were done. The associations of thyroid function with the risk of hypertensive disorders were studied.

MAIN OUTCOME MEASURES Mean blood pressures and hypertensive disorders, including pregnancy-induced hypertension (PIH; n=209) and preeclampsia (n=136).

RESULTS Hyperthyroid mothers had a higher risk of hypertensive disorders (OR (95% CI)=3.40 (1.46-7.91), *P*=0.005), which was mainly due to an increased risk of PIH (OR=4.18 (1.57-11.1), *P*=0.004). Hypothyroidism and hypothyroxinemia were not associated with hypertensive disorders.

Within the normal range, high-normal FT4 levels were associated with an increased risk of hypertensive disorders (OR=1.62 (1.06-2.47), *P*=0.03), which was mainly due to an increased risk of preeclampsia (OR=2.06 (1.04-4.08), *P*=0.04). TPOAb status was not associated with hypertensive disorders.

CONCLUSIONS We show that biochemical hyperthyroidism and also high-normal FT4 levels during early-pregnancy are associated with an increased risk of hypertensive disorders. These data demonstrate that these associations are even seen for mild variation in thyroid function within the normal range.

INTRODUCTION

Hypertensive disorders, including pregnancy-induced hypertension (PIH) and (pre)eclampsia, are common during pregnancy with an estimated prevalence of 2-8%.[1-3] Various studies have shown that hypertensive disorders are a major cause of both maternal and fetal morbidity and mortality. Amongst others, complications may include renal failure, disseminated intravascular coagulation, cerebrovascular bleeding, intrauterine growth retardation, abruptio placentae, premature delivery and still births.[1,3]

Both hypo- and hyperthyroidism have been shown to have important vascular effects, including endothelial cell dysfunction.[4-8] Therefore, a number of studies have investigated the association between thyroid dysfunction and hypertensive disorders during pregnancy.[9-19] Most of these studies were of limited sample size, but a number of large studies have also been published on this topic in the last decade.[9-12,15,16,19] Some of these studies have found an increased risk of hypertensive disorders in mothers with hypothyroidism[9,15,19] or hyperthyroidism[15], while others did not find any associations [10-12,16]. Differences between these studies might have been due to the fact that not all studies controlled for potentially confounding factors, such as thyroid autoimmunity, smoking, body mass index (BMI), ethnicity, socio-economic status, and parity.

More recently, a number of reports have shown that even minor variations in thyroid function can have important effects on pregnancy complications.[20-22] In this context it is interesting to note that none of these studies have investigated the associations between variation in thyroid function within the normal range and the risk of hypertensive disorders.

For these reasons, we studied the associations between thyroid function within the normal range, thyroid dysfunction, thyroid autoimmunity, and blood pressure, as well as the risk of hypertensive disorders during pregnancy. This study was carried out in a population-based pregnancy cohort including 5153 women, taking the effects of a wide range of confounding factors into account. Thyroid dysfunction was defined as a biochemical diagnosis, based on pregnancy specific calculated reference ranges independent of clinical symptoms.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based non-interventional cohort study from early fetal life onwards in Rotterdam, the Netherlands, which has been described in detail previously.[23,24] Mothers with a delivery date between April 2002 and January 2006 were enrolled. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all adult participants. 8880 women were enrolled in pregnancy in the Generation R Study. TSH and FT4 levels were determined in 5803 pregnant women. Women with twin pregnancies (n = 128), pre-existing thyroid disease (n = 81), thyroid (interfering) medication usage (n = 4) and fertility treatment (n = 68) were excluded. If subsequent pregnancies were recorded in the database only the record of the first pregnancy was used (n = 369 excluded). In total, 5153 women were included in one or more analyses.

Thyroid measurements

As part of the study, maternal serum samples were obtained in early pregnancy (mean (SD): 13.5 (2.0) wk).[24] Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics,

Rochester, NY), as described in detail previously. Reference ranges for TSH and FT4 levels were 0.03 – 4.03 mU/L and 10.4 – 21.9 pmol/L, respectively. Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when >60 IU/ml.[24]

Iodine measurements

Maternal serum and urinary samples were obtained at the same time (mean (SD): 13.5 (2.0) wk). Urinary iodine concentrations were determined in a random subset of 1085 women, which has been described in detail previously [25]. Median urinary iodine excretion was used to determine population iodine status as advocated by the WHO (with <150 µg/L as insufficient, 150 – 249 µg/L as adequate, and >500 µg/L as excessive).[26]

Hypertensive disorders

Blood pressure measurements were performed in early, mid and late pregnancy.[27] All participants were seated in upright position with back support, and were asked to relax for 5 minutes. A cuff was placed around the nondominant upper arm, which was supported at the level of the heart, with the bladder midline over the brachial artery pulsation. In case of an upper arm exceeding 33 cm a larger cuff (32–42 cm) was used. The mean value of two blood pressure readings over a 60 seconds interval was documented for each participant.

After each participant had given birth, the attending community midwife or obstetrician completed a delivery report. The reports on those participants who had given birth under the medical supervision of an obstetrician were selected and screened by a trained medical-record abstractor. To confirm the presence of gestational hypertension, the same abstractor conducted detailed reviews of these women's hospital charts and defined PIH or preeclampsia according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[28] Briefly, the following criteria were used to identify women with PIH: development of systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mmHg (at least two blood pressure readings) after 20 weeks of gestation in a previously normotensive woman. These criteria plus the presence of proteinuria (defined as two or more dipstick readings of 2+ or greater, one catheter sample reading of 1+ or greater, or a 24-hour urine collection containing at least 300 mg of protein) were used to identify women with preeclampsia.[29]

Covariates

Ultrasound measurements were used to establish gestational age in early pregnancy (gestational age 13.5 (2.0) wks).[23] Information on maternal age, parity, smoking status, socio-economic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status.[23] At enrollment, maternal height and weight were measured to calculate body mass index (BMI, kg/m^2).

Statistical analysis

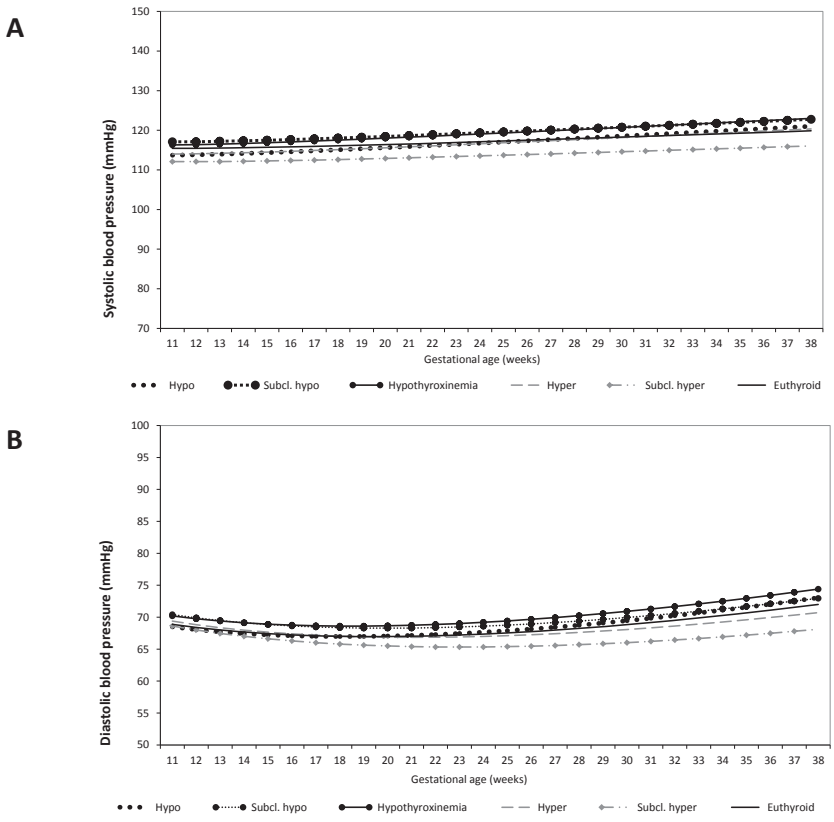
Reference ranges were determined by the 2.5th-97.5th percentiles, as described previously.[24] Hyperthyroidism was biochemically defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentiles) FT4; hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4, and hypothyroxinemia as a low FT4 with a normal TSH. The women that we identified as biochemical hypo- or hyperthyroidism in this study, were not treated for their thyroid dysfunction, as

serum measurements were performed after pregnancy in stored material. For the normal range TSH and FT4 quintiles, cut-off levels were: TSH: 1st 0.03-0.76 mU/L; 2nd 0.77-1.13 mU/L; 3rd 1.14-1.54 mU/L; 4th 1.55-2.12 mU/L; 5th 2.13-4.03 mU/L; and FT4: 1st 10.4-12.8 pmol/L; 2nd 12.9-14.1 pmol/L; 3rd 14.2-15.4 pmol/L; 4th 15.5-17.0 pmol/L; 5th 17.1-21.9 pmol/L.

Blood pressure levels and their course during pregnancy were analyzed in these groups and compared to the euthyroid group (i.e., women with normal TSH and FT4 levels) using a mixed linear model for repeated measurements which allowed for missing data points (in total 14,125 measurements performed).[30,31] For the normal range quintile analyses, the 3rd quintile was used as the reference quintile. Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Logistic regression analyses were used to calculate the risk of hypertensive disorders in these groups.

Analyses were adjusted for gestational age at venous puncture and blood pressure measurement, maternal age, smoking status, SES, parity, ethnicity, BMI and child gender. We used multiple imputation for covariates with missing data. Five imputed data sets were created and pooled for analyses. Smoking, SES, ethnicity (missing due to non-response in 13.0%, 7.1% and 5.7%, respectively), gestational age at blood sampling, and BMI (missing due to not recorded in 2.0% and <1.0%) were added to the model. Furthermore, we added hypertensive disorders, TSH, FT4 and TPOAb levels, maternal age, parity and child gender to the model as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. Women with pre-existing hypertension were excluded from analyses on hypertensive disorders, PIH or blood pressure. All analyses were repeated using reference ranges defined by the 5th-95th percentiles. Unless stated otherwise, statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

FIGURE 1. *Thyroid dysfunction and mean systolic (A) and diastolic (B) blood pressures during pregnancy.*



Differences between groups were small and not statistically significant.

RESULTS

The study population consisted of 5153 women, of which 345 (6.7%) developed a hypertensive disorder. Of these women, 209 (4.1%) developed PIH and 136 (2.6%) developed preeclampsia. Baseline characteristics of the studied population are shown in Table 1. Median urinary iodine excretion was 221 µg/L, indicating an iodine sufficient population.[26]

Thyroid dysfunction, normal range function and mean blood pressure during pregnancy.

Figure 1 shows the mean systolic and diastolic blood pressures during pregnancy for the biochemically determined thyroid dysfunction and euthyroid groups. The mean blood pressures for the normal-range TSH and FT4 quintiles are shown in Supplemental Figure 1. Minor differences in the course of blood pressures were found among the different groups, but this did not result in significant differences in mean blood pressures, except for a small difference in diastolic blood pressures between the fifth and third FT4 quintiles ($P_{adjusted}$ = 0.005).

Thyroid dysfunction, normal range function and risk of hypertensive disorders.

The relations between thyroid dysfunction, normal range thyroid function and hypertensive disorders are shown in Table 2. Women with hyperthyroidism had a 3.4-fold higher risk of developing a hypertensive disorder. The other studied thyroid dysfunction groups were not associated with hypertensive disorders. Within the normal range, women with high-normal FT4 levels also had an increased risk of hypertensive disorders. No significant associations were found with normal-range TSH levels.

Table 3 shows the associations between thyroid dysfunction, normal range thyroid function, and PIH as well as preeclampsia, when analyzed separately. Women with overt hyperthyroidism had a 4.2-fold higher risk of PIH. These women also seemed to have a higher risk of preeclampsia, but this effect was not statistically significant (*P* = 0.11). Within the normal range, high-normal FT4 levels were associated with a 2.1-fold increased risk of preeclampsia. There were no associations between normal-range TSH levels and PIH or preeclampsia.

When analyzing TSH and FT4 levels continuously over the entire range, FT4 but not TSH was associated with a borderline significant increased risk of PIH (FT4: OR = 1.03 (95%CI 1.00-1.07), *P* = 0.05; TSH: OR = 0.94 (95%CI 0.84-1.06), *P* = 0.32). There were no significant associations with the total group of hypertensive disorders (TSH: OR = 0.99 (95%CI 0.92-1.08), *P* = 0.85; FT4: OR = 1.03 (95%CI 1.00-1.06), *P* = 0.06) or preeclampsia (TSH: OR = 1.05 (95%CI 0.95-1.16), *P* = 0.33; FT4: OR = 1.01 (95%CI 0.97-1.05), *P* = 0.55). There were no associations between TPOAb-positivity and PIH or preeclampsia (data not shown).

Finally, similar results were obtained when reference ranges were defined by the 5th-95th percentiles. Thyroid (dys)function was not associated with maternal comorbidities that may influence the risk of hypertensive disorder including diabetes (N=50 gestational and N=20 pre-existing cases), hypercholesterolemia (N=26), chronic heart disorder (N=52), and systemic lupus erythematosus (N=1). Similar results were obtained when women with these comorbidities were excluded.

TABLE 1. *Characteristics of 5153 pregnant women from the Generation R Study*

Maternal age (yrs, mean (SD))	29.7 (5.1)
Thyroid function parameters[a] (median, 95% range)	
TSH (mU/L)	1.34 (0.03-4.03)
FT4 (pmol/L)	14.8 (10.4-21.9)
TPOAb-positivity (%)	5.5
Urinary Iodine excretion[a] (µg/L, median, 95% range)	221 (40-768)
Hypertensive disorder[b] (N (%))	345 (6.7)
Pregnancy induced hypertension (N (%))	209 (4.1)
Preeclampsia[c] (N (%))	136 (2.6)
BMI (kg/m^2, mean (SD))	24.5 (4.4)
Parity (%)	
Nullipara	60.9
Primipara	26.0
Multipara	13.1
Smoking (%)	
Never	72.6
Former	9.4
Active	18.0
Socio-economic status (%)	
Low	10.9
Middle	46.9
High	42.2
Ethnicity (%)	
Dutch	51.1
Moroccan	6.3
Turkish	8.7
Surinamese/Antillean	12.1
Other western	11.9
Other non-western	9.9
Child gender (% boys)	50.8

[a] *Blood and urine samples were collected at 13.5 (2.0) (mean (SD)) wks*
[b] *Includes pregnancy induced hypertension, preeclampsia, eclampsia and HELLP.*
[c] *Includes preeclampsia, eclampsia and HELLP.*

TABLE 2. *Thyroid dysfunction, normal range thyroid function, and the risk of hypertensive disorders during pregnancy.*

	Hypertensive disorders		
	% (N)	**OR (95% CI)**	**P**
Overt hypothyroidism	0.0 (0/17)	*NA*	*NA*
Subclinical hypothyroidism	8.5 (14/165)	1.23 (0.69-2.22)	0.47
Hypothyroxinemia	6.4 (9/129)	1.08 (0.53-2.22)	0.83
Overt hyperthyroidism	13.7 (7/51)	3.40 (1.46-7.91)	0.005
Subclinical hyperthyroidism	3.2 (2/62)	0.80 (0.19-3.34)	0.76
Euthyroidism[a] (reference)	6.2 (276/4451)	reference	
Normal range TSH			
1st quintile	6.8 (60/885)	1.36 (0.91-2.03)	0.14
2nd quintile	4.7 (43/907)	0.87 (0.56-1.33)	0.51
3rd quintile (reference)	5.6 (50/894)	reference	
4th quintile	6.3 (56/884)	1.11 (0.74-1.67)	0.61
5th quintile	7.6 (67/881)	1.23 (0.83-1.81)	0.31
Normal range FT4			
1st quintile	6.6 (59/897)	1.28 (0.83-1.96)	0.26
2nd quintile	6.6 (63/956)	1.36 (0.90-2.07)	0.15
3rd quintile (reference)	4.7 (40/848)	reference	
4th quintile	5.8 (51/885)	1.28 (0.83-1.98)	0.27
5th quintile	7.3 (63/865)	1.62 (1.06-2.47)	0.03

The thyroid dysfunction groups were defined biochemically, and not based on a clinical diagnosis of hypo- or hyperthyroidism. All analyses adjusted for gestational age at blood sampling, maternal age, BMI, smoking, SES, parity, ethnicity, and child gender. The %(N) column indicates the risk of a hypertensive disorder for the thyroid dysfunction group. The group of hypertensive disorders included both cases of pregnancy-induced hypertension (PIH) and preeclampsia.

[a] Defined as mothers with normal range (2.5th-97.5th percentiles) TSH and FT4 levels.

NA: Not available (no statistics were performed on this group as the number of persons with overt hypothyroidism was low and this group did not include any cases with hypertensive disorders).

DISCUSSION

In the current study, we investigated the associations between normal-range thyroid function, thyroid dysfunction and the risk of hypertensive disorders during pregnancy. We found that hyperthyroidism, as well as high-normal FT4 levels were associated with an increased risk of hypertensive disorders. The thyroid dysfunction groups were defined biochemically, and not based on a clinical diagnosis of hypo- or hyperthyroidism. We first studied the associations between thyroid (dys)function and mean systolic and diastolic blood pressure levels, but differences were small and not statistically significant. Also within the normal range no associations were observed, except for small differences in diastolic blood pressures between the fifth and third FT4 quintiles, ranging from 0-1 mmHg. Potential treatment of women with hypertensive disorders could have influenced these results on blood pressure levels during pregnancy. However, similar results were obtained after exclusion of this group. Although several studies have investigated the associations between thyroid dysfunction and hypertensive disorders during pregnancy[9-19], no data were so far available on the effects of variation in thyroid function within the normal range on the risk of hypertensive disorders during pregnancy. This study is therefore the first to demonstrate that also within the normal range, women with high-normal FT4 levels have an increased risk of hypertensive disorders during pregnancy. Stratified analyses showed that this effect was mainly driven by a 2-fold increased risk of preeclampsia, illustrating the importance of stratifying pregnancy related hypertensive disorder analyses for the exact disorder subtype.

TABLE 3. *Thyroid dysfunction, normal range thyroid function, and the risk of pregnancy induced hypertension or preeclampsia.*

	Pregnancy induced hypertension			Preeclampsia[a]		
	% (N)	OR (95% CI)	P	% (N)	OR (95% CI)	P
Overt hypothyroidism	0.0 (0/17)	NA	NA	5.6 (1/18)	1.84 (0.23-14.7)	0.56
Subclinical hypothyroidism	4.2 (7/165)	0.90 (0.41-2.00)	0.80	4.8 (8/168)	1.80 (0.85-3.82)	0.13
Hypothyroxinemia	4.7 (6/129)	1.23 (0.51-2.93)	0.65	3.8 (5/133)	1.29 (0.50-3.29)	0.60
Overt hyperthyroidism	9.8 (5/51)	4.18 (1.57-11.1)	0.004	5.7 (3/53)	2.68 (0.80-9.00)	0.11
Subclinical hyperthyroidism	1.6 (1/62)	0.72 (0.10-5.31)	0.75	1.6 (1/64)	0.64 (0.09-4.78)	0.66
Euthyroidism[b] (reference)	4.0 (177/4451)	reference		2.5 (113/4527)	reference	
Normal range TSH						
1st quintile	3.8 (34/892)	1.19 (0.71-1.98)	0.51	3.3 (30/905)	1.58 (0.89-2.81)	0.12
2nd quintile	3.7 (33/900)	1.11 (0.66-1.85)	0.70	1.4 (13/916)	0.62 (0.31-1.25)	0.18
3rd quintile (reference)	3.5 (31/894)	reference		2.3 (21/907)	reference	
4th quintile	3.7 (33/884)	1.06 (0.64-1.74)	0.84	2.9 (26/900)	1.22 (0.68-2.20)	0.51
5th quintile	5.2 (46/881)	1.33 (0.83-2.16)	0.24	2.6 (23/899)	1.01 (0.55-1.86)	0.97
Normal range FT4						
1st quintile	4.0 (36/897)	1.03 (0.62-1.72)	0.91	2.8 (26/915)	1.68 (0.85-3.34)	0.14
2nd quintile	4.1 (39/956)	1.11 (0.67-1.83)	0.68	2.7 (26/974)	1.68 (0.85-3.31)	0.14
3rd quintile (reference)	3.5 (30/848)	reference		1.5 (13/862)	reference	
4th quintile	3.7 (33/885)	1.04 (0.62-1.74)	0.89	2.5 (22/897)	1.81 (0.90-3.65)	0.10
5th quintile	4.5 (39/865)	1.25 (0.75-2.06)	0.39	3.0 (26/879)	2.06 (1.04-4.08)	0.04

The thyroid dysfunction groups were defined biochemically, and not based on a clinical diagnosis of hypo- or hyperthyroidism.
All analyses adjusted for gestational age at blood sampling, maternal age, BMI, smoking, SES, parity, ethnicity, and child gender.
The %(N) column indicates the risk of the respective hypertensive disorder for the thyroid dysfunction group.
[a] Includes preeclampsia, eclampsia and HELLP. [b] Defined as mothers with normal range (2.5th-97.5th percentiles) TSH and FT4 levels.
NA: Not available (no statistics were performed on this group as the number of persons with overt hypothyroidism was low and this group did not include any cases with hypertensive disorders).

Most of the studies that investigated the associations between thyroid dysfunction and hypertensive disorders had a limited sample size and showed conflicting results.[9-19] The largest study, recently published by Mannisto *et al.*[15], investigated the associations between hypo- or hyperthyroidism and hypertensive disorders in a retrospective US cohort of 223,512 pregnancies. An increased risk of preeclampsia was found for both hypo-and hyperthyroidism. Unfortunately, this study lacked information on treatment of thyroid disease during pregnancy and no data on TPOAb status were available. As data were derived from electronic medical records, the authors were not able to study more subtle alterations in thyroid function, including subclinical hypo- and hyperthyroidism and variation in thyroid function within the normal range. In a prospective population-based cohort of 24,883 pregnancies, Wilson *et al.* found positive associations between subclinical hypo- and hyperthyroidism and the risk of PIH, mild preeclampsia and severe preeclampsia.[19] However, after adjustment for confounding factors (i.e., maternal age, weight, ethnicity, and parity) the only remaining significant association was between subclinical hypothyroidism and severe preeclampsia. Ashoor *et al.* compared serum thyroid parameters in the first trimester between pregnant women that did or did not develop preeclampsia.[9] Although higher TSH and lower FT4 levels were found in 77 pregnant women that would later develop preeclampsia, no data were reported on the prevalence of overt hypothyroidism, subclinical hypothyroidism or hypothyroxinemia in these groups.

In contrast to the studies discussed above, a number of large studies did not find any associations between thyroid dysfunction and hypertensive disorders during pregnancy.[10-12,16] These conflicting

results could at least partially be due to the fact that not all studies had data on thyroid medication and various serum thyroid parameter cut-off levels were used to define thyroid disease. In addition, only part of these studies were able to correct for factors which are known to be associated with thyroid parameters and/or the risk of hypertensive disorders, including maternal BMI, age, parity, smoking, SES and ethnicity. The current population-based study investigates the associations between the entire range of thyroid (dys)function and blood pressure as well as hypertensive disorders during pregnancy. As thyroid function reference ranges can differ between populations [24], we calculated reference ranges in our own population and in our analyses we took a wide range of potentially interfering factors into account. In this way, we found that pregnant women with hyperthyroidism have a substantially increased risk of hypertensive disorders (13.7 vs 6.2%), mainly due to an increased risk of PIH. This group may include women with a slightly increased thyroid function due to high hCG levels, as well as women with Graves' disease. In our population-based study design, the number of cases are too low to stratify analyses for these subgroups. Furthermore, when analyzing TSH and FT4 levels over the entire range, we only detected a borderline significant positive association between FT4 and the risk of PIH.

This can be explained from the results of Table 2, as within the normal range the lowest and highest FT4 and TSH quintiles had an increased risk of hypertensive disorders compared to the middle quintile, although not all statistically significant. Outside the normal range, both hyperthyroid and subclinical hypothyroid mothers had an increased risk of hypertensive disorders compared to euthyroid mothers. Although our study included more than 5,000 pregnant women, there were only few cases with overt hypothyroidism, limiting the statistical power for this group. This was also the case when analyzing the associations between overt hyperthyroidism and preeclampsia only (Table 3). Another limitation of the current study is that only early pregnancy (mean = 13.5 wks) TSH and FT4 levels were available. We therefore do not know if all women in the identified high-risk groups remained biochemically hyperthyroid or had high-normal FT4 levels during the entire pregnancy. To our knowledge, there is only one large study correlating TSH and FT4 levels in various trimesters of pregnancy.[32] Lambert-Messerlian *et al.* found a strong positive correlation between TSH levels in the first and second trimesters of pregnancy (r^2=0.64), whereas the correlations for FT4 levels were also positive but lower (r^2 = 0.23).[32] As there are no other studies correlating TSH and FT4 levels throughout pregnancy, more large studies are needed which also take the third trimester into account.

Hypertensive disorders during pregnancy are of great importance as they account for 16% of the worldwide maternal deaths.[2] Not only have these disorders been associated with an increased risk of maternal and child morbidity and mortality during pregnancy, but also after pregnancy. For example, various studies have shown an increased risk of maternal hypertension, ischemic heart disease, stroke, end-stage renal disease, and mortality in later life[32-34], as well as an increased risk of childhood hypertension, cognitive limitations, and mortality.[3,34-37]

Possible mechanisms by which thyroid hormone may influence the onset of hypertensive disorders during pregnancy come from studies which investigated the cardiovascular effects of thyroid dysfunction. Some of these studies have shown that (subclinical) hypothyroidism is associated with increased vascular resistance, increased blood pressure, ventricular hypertrophy and endothelial cell dysfunction, characterized by decreased nitric oxide production with impaired vasorelaxation.[6,8] Although less is known about the vascular effects of high-normal FT4 levels or hyperthyroidism, a few studies have shown that patients with Graves' hyperthyroidism have a reduction in protective mechanisms against endothelial damage, and show signs of endothelial cell activation and dysfunction.[4,5,7,38] These studies suggest that high thyroid hormone levels can lead to endothelial cell dysfunction, which is known to play a pivotal role in the pathophysiology of hypertensive disorders in pregnancy.[3] However, the exact mechanisms underlying the associations between high-normal thyroid function, hyperthyroidism and hypertensive disorders during pregnancy need to be clarified in future studies.

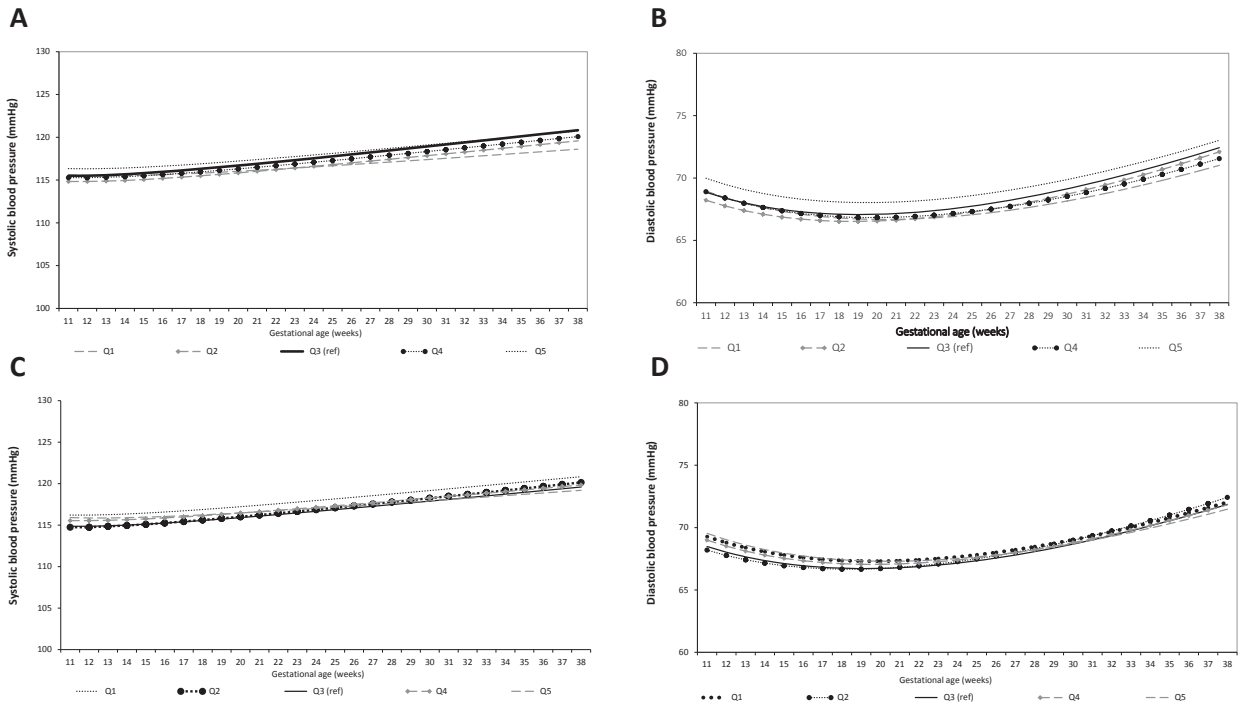
Given the wide range of detrimental effects of hypertensive disorders during pregnancy, various studies have tried to identify risk factors in early pregnancy for the development of hypertensive disorders, and it is remarkable to note that only few risk factors have been identified.[3] The current study identifies high-normal FT4 levels and hyperthyroidism during early pregnancy as risk factors for hypertensive disorders. To predict which mothers will develop hypertensive disorders during pregnancy, Poon et al. developed a prediction model, including maternal history, uterine artery pulsatility index, mean arterial pressure, pregnancy-associated plasma protein-A and placental growth factor.[39] Future studies should analyze if serum thyroid function tests could increase the sensitivity of this prediction model. Furthermore, given that only few risk factors have been associated with the development of hypertensive disorders during pregnancy, the diagnostic workup after the diagnosis of a hypertensive disorder is limited.[3] Our results suggest that it would be useful to add thyroid function testing to this diagnostic workup.

However, it is important to note that there are only a limited number of large studies with data on various potential confounders which have investigated the associations between thyroid (dys)function and the risk of hypertensive disorders during pregnancy. Therefore, our results should first be replicated in an independent population. Furthermore, there is no evidence at this stage that women at risk for hypertensive disorders would benefit from treatment with antithyroid drugs.

In conclusion, we show that hyperthyroidism and also high-normal FT4 levels during early-pregnancy are risk factors for the development of hypertensive disorders. These data demonstrate that even mild variation in thyroid function within the normal range can have such effects.

APPENDIX

SUPPLEMENTAL FIGURE 1. *Normal-range TSH and FT4 quintiles and mean systolic (a+c) and diastolic (b+d) blood pressures during pregnancy.*



Besides a significant difference in diastolic blood pressures between FT4-Q5 and -Q3 (Padjusted = 0.005), differences were small and not statistically significant. TSH Quintiles: Q1:0.03-0.76 mU/L; Q2: 0.77-1.13 mU/L; Q3: 1.14-1.54 mU/L; Q4: 1.55-2.12 mU/L; Q5: 2.13-4.03 mU/L. FT4 Quintiles: Q1: 10.4-12.8 pmol/L; Q2: 12.9-14.1 pmol/L; Q3: 14.2-15.4 pmol/L; Q4: 15.5-17.0 pmol/L; Q5: 17.1-21.9 pmol/L.

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CHAPTER 11

HYPOTHYROXINEMIA AND TPO-ANTIBODY POSITIVITY ARE RISK FACTORS FOR PREMATURE DELIVERY

Tim I. M. Korevaar, Sarah Schalekamp-Timmermans, Yolanda B. de Rijke, W. Edward Visser, Willy Visser, Sabine M. P. F. de Muinck Keizer-Schrama, Albert Hofman, H. Alec Ross, Herbert Hooijkaas, Henning Tiemeier, Jacoba J. Bongers-Schokking, Vincent W. V. Jaddoe, Theo J. Visser, Eric A. P. Steegers, Marco Medici and Robin P. Peeters

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ABSTRACT

CONTEXT Premature delivery is an important risk factor for child mortality and psychiatric, metabolic and cardiovascular disease later in life. In the majority of cases, the cause of prematurity cannot be identified. Currently, it remains controversial if an abnormal maternal thyroid function during pregnancy increases the risk of a premature delivery. Therefore, we investigated the relation between maternal serum thyroid parameters and the risk of premature delivery in a large prospective population-based study.

DESIGN Serum TSH, FT4, T4 and TPO-antibodies (TPOAb) were determined during early pregnancy in 5971 pregnant women from the Generation R study. Data were available on maternal age, parity, smoking, socio-economic status, ethnicity, maternal anthropometrics and urinary iodine levels.

RESULTS Of all women, 5.0% had a premature delivery (<37 weeks), 4.4% had a spontaneous premature delivery and 1.4% a very premature delivery (<34 weeks). High TSH levels and subclinical hypothyroidism were associated with premature delivery but not with spontaneous premature delivery.

Maternal hypothyroxinemia was associated with a 2.5-fold increased risk of premature delivery, a 3.4-fold increased risk of spontaneous premature delivery and a 3.6-fold increased risk of very premature delivery (all $P<0.01$).

TPOAb positivity was associated with a 1.7-fold increased risk of premature delivery ($P=0.01$), a 2.1-fold increased risk of spontaneous premature delivery ($P=0.02$) and a 2.5-fold increased risk of very premature delivery ($P=0.04$). These effects remained similar after correction for TSH/FT4 levels.

CONCLUSIONS Hypothyroxinemia and TPOAb positivity are associated with an increased risk of premature delivery. The increased risk in TPOAb positive women seems to be independent of thyroid function.

INTRODUCTION

Premature delivery has been identified as a risk factor for psychiatric, metabolic, cardiovascular and renal disease later in life.[1-3] Furthermore, it has been identified as the largest direct cause of child deaths in almost all high and middle-income countries.[4] In 2010, the estimated incidence of premature deliveries in developed countries was 5-12%, yet in the majority of these women no known risk factors can be identified.[5,6] Severe hypo- and hyperthyroidism during pregnancy are associated with premature delivery, but conflicting results have been published on milder alterations in thyroid function tests over the last two decades.[7,8] To date, most studies could not investigate spontaneous deliveries, even though this is a more homogeneous group which much better represents the physiology of prematurity.

Two studies have demonstrated a relation between increased TSH levels and a higher risk of premature delivery [9,10], but both studies lacked data on FT4 or TPOAbs. In contrast, Negro *et al*. and Allan *et al*. did not find an increased risk of prematurity in women with a high TSH level, but the definition of an increased TSH level varied between these studies.[11,12] Conflicting results on the relation between subclinical hypothyroidism (elevated TSH and normal FT4) during pregnancy and premature delivery have been published as well.[13-18] Whether hypothyroxinemia (normal TSH with low FT4) increases the risk of premature delivery has only been investigated by three groups.[14,16,19] Even though hypothyroxinemia was not associated with premature delivery, a study by Cleary-Goldman *et al*. found that hypothyroxinemia in the first, but not in the second trimester was associated with premature onset of labor.

TPOAb positivity is generally accepted as a risk factor for prematurity [18,20-23], although not all studies could confirm this association [17,19,24,25]. It is still unknown whether the possible increased risk of prematurity in TPOAb positive women is due to an effect on the thyroid or a direct effect of autoimmunity itself, as no study has investigated TPOAb positivity as a risk factor for prematurity independent of thyroid status.

As a consequence, it remains controversial whether milder forms of maternal thyroid dysfunction during pregnancy are associated with premature delivery. Therefore, we investigated the relation between abnormal maternal thyroid function during early pregnancy and the risk of a premature and spontaneous premature delivery in a large prospective population-based study. In these analyses, we included a wide variety of possible interfering factors, and additionally studied the (independent) effect of TPOAb status on premature delivery.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[26]

Population for analyses

Data on early pregnancy TSH and/or TPOAb levels and gestational age at birth were available for 6264 pregnant women. Women with twin pregnancies (N=128), pre-existing thyroid disease (N=85), thyroid (interfering) medication usage (N=4) and fertility treatment (N=76) were excluded. The final population comprised of 5971 women which were included in one or more analyses, of which 5622 women had available data on FT4 levels.

Birth outcomes

Prematurity was defined as a gestational age at birth <37 weeks and very premature delivery was defined as a gestational age at birth <34 weeks. Spontaneous (very) premature was defined as a spontaneous onset of premature labor before the 37th or 34th week of gestation and included women that did not deliver after induction of labor or by an elective caesarean section. Premature rupture of membranes was defined as ruptured membranes before 37 weeks' gestation.

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks; 95% range 9.6-17.6). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and regarded as positive when >60 IU/ml.[27]

Iodine measurements

Urinary iodine concentrations were determined in a random subset of 1099 women during early pregnancy (median = 12.9 weeks; 95% range 9.8-17.2) as previously described.[28]

Covariates

Analyses were adjusted for known determinants of thyroid function and gestational age at birth. Gestational age was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[26] Information on maternal age, smoking status, socio-economic status (SES) and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands.[26] Surinamese women were defined as Creole, Hindustani or other, whereas Moroccan women were defined as Berber, Arabic or undefined Moroccan.[29] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. SES was defined by educational level, net household income, and employment status.[26] Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). Information on fertility treatment, pregnancy outcome, date of birth, birth anthropometrics, and the sex of the child were obtained from community midwives, obstetricians, and hospital registries.

Statistical analysis

Reference ranges were determined by population based calculations, as previously described.[27] Hyperthyroidism was defined as a low (<2.5th percentile) TSH with a high (>97.5th percentile) FT4, hyperthyroidism during pregnancy should be considered as a more biochemical diagnosis compared to hyperthyroidism in a non-pregnancy state; subclinical hyperthyroidism as a low TSH with a normal (2.5th – 97.5th percentiles) FT4; hypothyroidism as a high TSH with a low FT4; subclinical hypothyroidism as a high TSH with a normal FT4 and hypothyroxinemia as a low FT4 with a normal TSH. We studied the risk of premature delivery in these women, with the euthyroid women (i.e., women with normal TSH and FT4 levels) as the reference group.

The Endocrine Society and American Thyroid Association (ATA) guidelines recommend the use of population-based trimester-specific reference ranges. When population based reference ranges are unavailable upper limits for TSH are >2.5 mU/L in the first, or >3.0 mU/L in the second trimester are recommended.[7,8] In this paper the term high TSH has been subdivided according to these recommendations, references are made throughout.

To achieve a normal distribution, TSH values were logarithmically transformed. Intrauterine growth retardation (IUGR) is a major cause of iatrogenic prematurity and may be an intermediate between thyroid function and gestational age at birth. In this study, IUGR was defined by small for gestational age at birth (SGA; defined as a gestational age-adjusted birth weight below the 2.5th percentile in the study cohort (less than 2.13 SD)). However, sensitivity analysis showed that correction for SGA did not influence our analyses and therefore SGA was not adjusted for.

Women with comorbidities (including pre-existing diabetes, chronic hypertension, hypercholesterolemia, chronic heart disorder, systemic lupus erythematosus, and preeclampsia) were additionally excluded in all analyses as a number of studies have shown that these women may have higher TSH levels and/or a higher prevalence of TPOAb positivity and prematurity. This is line with our study, in which women with comorbidities had higher mean TSH (1.86 *vs.* 1.59 mU/L in women without comorbidities), showed a trend towards increased prevalence of TSH >97.5th percentile (adjusted odds ratio (aOR) 1.47 (0.87-2.48); *P*=0.14) and were more likely to have a premature delivery (aOR 4.54 (3.26-6.33); *P*<0.01).

Median urinary iodine excretion was used to determine population iodine status as advocated by the WHO (with <150 µg/L as insufficient, 150 – 249 µg/L as adequate, and >500 µg/L as excessive).[30] We used multiple imputation for covariates with >5.0% missing data. Five imputed data sets were created and pooled for analyses. Smoking, socio-economic status and ethnicity were added to the model (missing due to non-response in 12.8%, 7.2% and 5.7%, respectively). Furthermore, we added gestational age at birth, TSH, FT4 and TPOAb levels, maternal age, parity, fetal gender and maternal BMI to the model as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. All statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

RESULTS

The study population consisted of 5971 women of which 5.0% had a premature delivery (<37 weeks of gestation), and 1.4% had a very premature delivery (<34 weeks of gestation). Descriptive characteristics are shown in Table 1. The prevalence of TPOAb positivity was 5.6%, hypothyroidism 0.3%, subclinical hypothyroidism 3.4%, hyperthyroidism 1.0%, subclinical hyperthyroidism 1.4% and hypothyroxinemia 2.6%. Median urinary iodine excretion was 223 µg/L, indicating an iodine sufficient population.[30] No differences in urinary iodine status were seen between mothers of term newborns (median 223 µg/L), premature newborns (221 µg/L) or very premature newborns (248 µg/L) (*P*=0.83).

Elevated TSH levels, (subclinical) hypo- and hyperthyroidism and the risk of prematurity.

Table 2 shows the risk of prematurity for elevated TSH levels and (subclinical) hypo- and hyperthyroidism. Women with a TSH >97.5th percentile had an increased risk of premature and very premature delivery. A TSH level >97.5th percentile was no longer associated with premature delivery or very premature delivery after the exclusion of TPOAb positive women or after the exclusion of women with comorbidities. There was no association between high TSH and spontaneous (very) premature delivery. Current Endocrine Society and ATA guidelines recommend the use of an upper limit for TSH of 2.5 mU/L in the first- and of 3.0 mU/L in the second- and third trimester when population-based trimester-specific reference ranges are not available.[7,8] Women with elevated TSH levels according to these cut-off values also did not have an increased risk of a premature delivery. Similar results were found when only spontaneous deliveries were considered or when TPOAb positive women were excluded.

Compared to euthyroid women, women with subclinical hypothyroidism had an increased risk of premature and very premature delivery. Similar to the TSH levels >97.5th percentile analyses, subclinical hypothyroidism was no longer associated with premature delivery or very premature delivery when women with comorbidities were excluded. There was no association between subclinical hypothyroidism and spontaneous premature delivery. Women with overt hyperthyroidism or subclinical hyperthyroidism did not have an increased risk of premature delivery or spontaneous premature delivery.

TABLE 1. *Descriptive statistics of 5971 women.*

Maternal age	years (SD)	29.7	(5.0)
Gestational age at blood sampling	weeks (SD)	13.5	(2.0)
Gestational age at birth	weeks (SD)	39.9	(1.9)
Premature pregnancies <37 weeks	(N (%))	299	(5.0)
Spontaneous premature pregnancies	(N (%))	196	(4.4)
Very premature <34 weeks	(N (%))	83	(1.4)
Spontaneous premature pregnancies	(N (%))	41	(0.9)
Thyroid parameters	**(median)**		
TSH	(mU/L)	1.35	
FT4	(pmol/L)	14.8	
T4	(nmol/L)	145	
TPOAb positivity	(N (%))	312 (5.6)	
Parity	(N (%))		
Nullipara		3399	(57.4)
Primipara		1763	(29.8)
Multipara		757	(12.8)
Smoking*	(N (%))		
Non-smokers		4380	(73.4)
Stopped smokers		546	(9.1)
Smokers		1045	(17.5)
Socio-economic status*	(N (%))		
Low		596	(10.0)
Middle		2723	(45.6)
High		2652	(44.4)
Ethnicity*	(N (%))		
Dutch		3169	(53.1)
Moroccan		348	(5.8)
Turkish		473	(7.9)
Antillean		177	(3.0)
Surinamese		511	(8.6)
Other western		723	(12.1)
Other non-western		570	(9.5)
Maternal body mass index	(Mean (SD))	24.5	(4.4)
Child gender	N (%) (boys/girls)	3009 (50.4)	2959 (49.6)
Urinary I excretion	(median, µg/L)	223	

Descriptive statistics of the study population after exclusion of women with twin pregnancies, pre-existing thyroid disease, thyroid (interfering) medication usage or fertility treatment.

Hypothyroxinemia and the risk of prematurity

As shown in Table 3, women with hypothyroxinemia had a 2.5-fold increased risk of premature delivery and a 3.6-fold increased risk of very premature delivery when all pregnancies were considered. A 3.4-fold increased risk of spontaneous premature delivery and a 4.2-fold increased risk of spontaneous very premature delivery were observed. Similar significant results were found when all women with low FT4 levels were analyzed irrespective of their TSH, as well as after the exclusion of TPOAb positive women or women with comorbidities (data not shown).

TPOAb positivity and the risk of prematurity

Table 4 displays the risk of premature delivery according to TPOAb status. TPOAb positivity was associated with a 1.7-fold increased risk of premature, a 2.5-fold increased risk of very premature, and a 2.1-fold increased risk of spontaneous premature delivery. In euthyroid subjects, TPOAb positivity was not associated with a (very) premature delivery. Compared to TPOAb negative women, TPOAb positive women had higher median TSH levels and lower mean FT4 levels (1.31 *vs*. 2.65 mU/L and 15.2 *vs*. 14.6 pmol/L, respectively; both $P<0.01$). To study whether the increased risk of prematurity in TPOAb positive women is driven through an effect on the thyroid, analyses were additionally adjusted for TSH and FT4. Results remained similar after correction for these serum thyroid function parameters.

The lower half of Table 4 shows the risk of a premature delivery in TPOAb positive women with a concomitant high TSH. For women with a TSH >2.5 mU/L in the first trimester or >3.0 mU/L in the second, a trend towards an increased risk of premature delivery was seen ($P=0.06$). However, no effects on spontaneous premature or very premature delivery were detected. Low FT4 values were not associated with increased risks of premature delivery in TPOAb positive women.

Abnormal thyroid parameters and premature rupture of membranes.

Thirty percent of all premature deliveries are related to premature rupture of membranes.[6] We extended our analyses to study whether abnormal thyroid parameters could also be a risk factor for premature rupture of membranes. We found that women with hypothyroxinemia had an increased risk of premature rupture of membranes, as is shown in Table 5. Similar significant results were found when all women with low FT4 levels were analyzed irrespective of their TSH, as well as after the exclusion of TPOAb positive women or women with comorbidities (data not shown).

TABLE 2. *High TSH, thyroid disease entities and the risk of prematurity.*

	Spontaneous and iatrogenic deliveries (N=5971)					Only spontaneous deliveries (N=4446)			
	Prematurity <37 weeks			Prematurity <34 weeks		Prematurity <37 weeks		Prematurity <34 weeks	
	Prematurity % (N)	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*
TSH >97.5th percentiles (>4.04 mU/l)	7.8 (17/217)	1.87 (1.11-3.14)	0.02[e]	2.46 (1.04-5.83)	0.04	1.28 (0.61-2.68)	0.52	0.74 (0.10-5.53)	0.77
Non-elevated TSH (reference)	4.7 (235/5370)								
TPOAb-positive women excluded	2.3 (3/128)	1.38 (0.66-2.89)	0.39	1.97 (0.60-6.47)	0.27	1.04 (0.37-2.89)	0.95	1.30 (0.17-9.89)	0.80
Elevated TSH (ENDO/ATA guidelines)[b]	5.4 (30/551)	1.15 (0.77-1.70)	0.50	1.31 (0.64-2.69)	0.46	1.02 (0.61-1.71)	0.95	0.58 (0.14-2.47)	0.46
Non-elevated TSH (reference)	5.0 (252/5036)								
TPOAb-positive women excluded	4.1 (16/386)	0.87 (0.52-1.47)	0.60	1.07 (0.42-2.73)	0.89	0.77 (0.39-1.54)	0.46	0.94 (0.22-4.03)	0.93
Hypothyroidism	5.3 (1/19)	1.08 (0.14-8.36)	0.94	4.29 (0.50-36.8)	0.18	-	-	-	-
Subclinical hypothyroidism	8.0 (15/188)	2.04 (1.17-3.56)	0.01[f]	2.62 (1.02-6.74)	0.05	1.58 (0.75-3.32)	0.23	0.91 (0.12-6.86)	0.93
Hyperthyroidism	3.6 (2/56)	0.66 (0.16-2.78)	0.57	-	-	1.02 (0.24-4.39)	0.98	-	-
Subclinical hyperthyroidism	6.5 (5/77)	1.39 (0.54-3.55)	0.49	1.07 (0.14-8.07)	0.95	0.38 (0.05-2.79)	0.34	-	-
Euthyroid[d] (reference)	4.7 (235/4970)								

[a] Analysis adjusted for gestational age at blood sampling, maternal age, smoking, SES, parity, ethnicity, maternal BMI, maternal height and child gender. [b] TSH >2.5 mU/L in the first,- or >3.0 in the second trimester (ATA and Endocrine Society guidelines). [c] Defined as a TSH > 4.3 mU/L. [d] Defined as TSH and FT4 levels within the 2.5th-97.5th percentiles. [e] aOR 1.62 (0.86-3.06); P=0.14 after exclusion of women with comorbidities. [f] aOR 1.54 (0.77-3.09); P=0.23 after exclusion of women with comorbidities. Disease parameters were calculated using TSH and FT4.

TABLE 3. *Hypothyroxinemia and the risk of prematurity.*

	Spontaneous and iatrogenic deliveries (N=5971)					Only spontaneous deliveries (N=4446)			
	Prematurity <37 weeks			Prematurity <34 weeks		Prematurity <37 weeks		Prematurity <34 weeks	
	Prematurity % (N)	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*
Hypothyroxinemia	10.3 (15/145)	2.54 (1.42-4.54)	<0.01	3.56 (1.50-8.43)	<0.01	3.44 (1.76-6.70)	<0.01	4.21 (1.34-13.3)	0.01
Euthyroid[b] (reference)	4.7 (235/4970)								

[a] Analysis adjusted for gestational age at blood sampling, maternal age, smoking, SES, parity, ethnicity, maternal BMI, maternal height and child gender. [b] Defined as TSH and FT4 levels within the 2.5th-97.5th percentiles.

TABLE 4. *Maternal TPOAb status and the risk of prematurity.*

	Spontaneous and iatrogenic deliveries (N=5971)					Only spontaneous deliveries (N=4446)			
	Prematurity <37 weeks			Prematurity <34 weeks		Prematurity <37 weeks		Prematurity <34 weeks	
	Prematurity % (N)	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*
Positive TPOAb status	7.4 (23/312)	1.70 (1.08-2.67)	0.02	2.49 (1.21-5.12)	0.01	2.05 (1.04-3.17)	0.04	2.21 (0.76-6.45)	0.15
Negative (reference)	4.7 (250/5264)								
Positive TPOAb status (in euthyroids[b])	5.9 (13/219)	1.31 (0.70-2.48)	0.40	2.29 (0.88-5.95)	0.09	1.31 (0.37-4.62)	0.68	2.69 (0.77-9.39)	0.12
Negative (reference)	4.6 (206/4467)								
Positive TPOAb status (adjusted for TSH/FT4[c])	7.5 (22/294)	1.70 (1.04-2.79)	0.04	2.24 (0.99-5.07)	0.05	1.82 (0.98-3.37)	0.06	1.87 (0.52-6.80)	0.34
Negative (reference)	4.7 (231/4869)								
Amongst TPOAb positive women only									
TSH >97.5th percentiles (>4.04 mU/l)	12.9 (9/70)	3.27 (1.15-9.29)	0.03	3.05 (0.50-18.6)	0.23	1.61 (0.39-6.67)	0.51	-	-
Non-elevated TSH (reference)	5.8 (13/225)								
Elevated TSH (ENDO/ATA guidelines[d])	10.4 (14/135)	2.67 (0.95-7.53)	0.06	1.85 (0.34-10.2)	0.48	1.34 (0.36-5.00)	0.67	-	-
Non-elevated TSH (reference)	5.0 (8/160)								
FT4 below the 2.5th percentile	8.7 (2/23)	1.46 (0.17-12.2)	0.72	2.34 (0.02-242)	0.71	1.11 (0.09-14.4)	0.94	-	-
Euthyroid[c] (reference)	5.4 (11/204)								

[a] Analysis adjusted for gestational age at blood sampling, maternal age, smoking, SES, parity, ethnicity, maternal BMI, maternal height and child gender. [b] Defined as TSH and FT4 levels within 2.5th-97.5th percentiles. [c] Analysis adjusted for gestational age at blood sampling, maternal age, smoking, SES, parity, ethnicity, maternal BMI, maternal height, child gender, TSH and FT4.
[d] TSH >2.5 mU/L in the first,- or >3.0 in the second trimester (ATA and Endocrine Society guidelines)
Analyses amongst TPOAb positive women only, for prematurity <34 weeks in only spontaneous deliveries are not displayed due to insufficient numbers.

TABLE 5. *Maternal thyroid parameters and premature rupture of membranes.*

	Spontaneous and iatrogenic deliveries (N=5971)				Only spontaneous deliveries (N=4446)	
	PROM <37 weeks				PROM <37 weeks	
	PROM % (N)		aOR (95%CI)[a]	*P*	aOR (95%CI)[a]	*P*
TSH >97.5th percentiles (>4.04 mU/l)	4.3	(9/209)	1.29 (0.65-2.59)	0.47	1.06 (0.46-2.48)	0.89
Non-elevated TSH (reference)	3.7	(175/4776)				
TPOAb-positive women excluded	2.3	(3/128)	0.71 (0.22-2.26)	0.56	0.56 (0.14-2.33)	0.43
Elevated TSH (ENDO/ATA guidelines)[b]	2.8	(190/4844)	0.71 (0.41-1.21)	0.21	0.63 (0.33-1.21)	0.16
Non-elevated TSH (reference)	3.9	(15/528)				
TPOAb-positive women excluded	2.2	(8/365)	0.57 (0.28-1.17)	0.13	0.56 (0.24-1.29)	0.80
Hypothyroidism	5.3	(1/19)	1.47 (0.19-11.3)	0.71	1.86 (0.23-15.0)	0.56
Subclinical hypothyroidism	4.4	(8/180)	1.35 (0.65-2.81)	0.42	1.01 (0.40-2.54)	0.98
Hyperthyroidism	1.8	(1/56)	0.42 (0.06-3.08)	0.39	0.52 (0.07-3.92)	0.53
Subclinical hyperthyroidism	2.7	(2/74)	0.72 (0.17-3.01)	0.65	-[c]	-
Euthyroid[d] (reference)	3.7	(175/4776)				
Hypothyroxinemia	7.2	(10/138)	2.35 (1.18-4.69)	0.02	2.74 (1.30-5.75)	<0.01
Euthyroid[b] (reference)	3.7	(175/4776)				
Positive TPOAb status	7.5	(22/294)	1.42 (0.82-2.45)	0.21	1.43 (0.75-2.71)	0.27
Negative (reference)	4.7	(231/4869)				

[a] Analysis adjusted for gestational age at blood sampling, maternal age, smoking, SES, parity, ethnicity, maternal BMI, maternal height and child gender. [b] Defined as TSH and FT4 levels within 2.5th-97.5th percentiles. [c] No PROM occurred in subclinical hyperthyroid women with a spontaneous delivery. PROM = premature rupture of membranes.

DISCUSSION

A premature delivery is associated with various adverse effects on child health and survival. Yet, in approximately half the cases a risk factor cannot be determined [6]. In the current study we demonstrate that pregnant women with low FT4 levels in the first or second trimester have an increased risk of a premature delivery, which is independent of their TSH, TPOAb status or concomitant comorbidities. Women with an elevated TSH and/or with subclinical hypothyroidism also have an increased risk of premature delivery but this association does not persist after exclusion of TPOAb positive women or women with comorbidities. Finally we show that TPOAb positivity is also associated with an increased risk of premature delivery and this association is independent of thyroid function.

In some studies an increased risk for premature delivery has been described amongst women with an elevated TSH [9,10] or with subclinical hypothyroidism [13-15] whereas other studies could not confirm these findings.[11,12,16-18] Different cut-offs for TSH have been used in different studies. As advocated by international guidelines, we used population based reference ranges because in practice, pre-determined cut-off values are prone to result in inter-observer errors due to methodological differences. In line with previous studies, our data showed an association of an elevated TSH and of subclinical hypothyroidism with an increased risk of a premature delivery. However, this correlation no longer persisted after exclusion of women with concomitant comorbidities and there was no association with

spontaneous premature delivery. Furthermore, the association between subclinical hypothyroidism and premature delivery disappeared after the exclusion of TPOAb positive women. This finding supports previous suggestions that the association with premature delivery is caused by a high prevalence of TPOAb positive women amongst women with subclinical hypothyroidism (32% in this study).[14] The ~50% reduction in risk after the exclusion of TPOAb positive women and/or women with comorbidities suggests independent direct effects on both thyroid function and premature delivery. Taken together, our data do not support the concept that subclinical hypothyroidism is an independent risk factor for premature delivery.

Limited data are available on the risk of prematurity in women with hypothyroxinemia. So far hypothyroxinemia has not been identified as a risk factor for premature delivery.[14,16,19] Hypothyroxinemia in the first trimester has been associated with premature labor (defined as persistent uterine contractions accompanied by cervical change on digital examination before 37 weeks of gestation).[16] The single study that focused on spontaneous deliveries found no difference in the prevalence of hypothyroxinemia (defined as FT4 <5th percentiles) between very premature and term deliveries even though women with a premature delivery in this particular study did have a lower median FT4 (0.94 *vs*. 0.99; *P*<0.001).[19] However, in the current study we demonstrate a clear association between hypothyroxinemia and both (very) premature and spontaneous (very) premature delivery which is independent of TPOAb positivity (8% amongst hypothyroxinemia group) or concomitant comorbidities. A possible explanation for this discrepancy may be a difference in iodine status between the studies, as low levels of FT4 due to iodine deficiency may be transient and/or have different consequences than other causes of hypothyroxinemia. In the current study we demonstrate that our population is iodine sufficient. The other studies with negative results were performed in the United States and the United Kingdom. Although these countries are generally considered iodine sufficient, borderline sufficient or even insufficient iodine status during pregnancy has been described in both countries.[31,32]

Data on hypothyroidism suggest that various pathways may be involved in the mechanism via which low FT4 levels increase the risk for prematurity. For example, oxytocin and vasopressin are known to play a role in the onset of labor [33,34] and elevated levels of vasopressin have been shown in hypothyroid women. Similarly, animal studies have shown that both vasopressin and oxytocin are elevated in hypothyroid rats.[35] Surfactant protein A (SP-A) may also play a role since animal studies have identified SP-A, as a hormone of parturition which modulates the intrauterine inflammatory response related to spontaneous premature labor.[36,37] SP-A is secreted by the lungs and high amounts of SP-A mRNA have been shown in the lungs of pups from hypothyroid mothers [38] whereas administration of T3 decreased the expression of SP-A genes in fetal rat lungs during late gestation.[39]

Even though not all studies point in the same direction [17,19,24,25], TPOAb positive pregnant women are generally considered to have a higher risk for premature delivery.[18,20-23] A recent meta-analysis showed that TPOAb positive euthyroid women have an increased risk of a delivery before 37 weeks of gestation, although the mechanism via which TPOAbs may cause premature births is poorly understood.[40] Our results confirm this association, and we additionally show that this effect is also present when only women with spontaneous deliveries are analyzed.

In order to investigate the mechanism behind the relation of TPOAbs and prematurity we additionally adjusted TPOAb analyses for serum TSH and FT4 levels. The fact that these results remained similar after correction for these markers of thyroid function suggests that the positive relation between TPOAbs and prematurity is independent of thyroid function and it is therefore likely to be due to the autoimmune process itself.

To date, this is one of the largest and most detailed studies investigating the effects of an abnormal maternal thyroid function during pregnancy on the risk of premature delivery. We discriminate

spontaneous from iatrogenic deliveries and investigate the entire range of serum thyroid abnormalities (including hypothyroxinemia and subclinical hypothyroidism). Important additional strengths are that we assessed the iodine status of our population, investigated a possible interfering role for comorbidities and IUGR, and corrected for a wide range of known determinants of premature delivery such as smoking, ethnicity, parity, maternal BMI and child gender. We also add valuable clinical data ruling out the possibility that TPOAb may cause premature delivery via alterations in thyroid function. This study was limited by the fact that we had a lower number of first trimester data. Analyses in overt hypothyroidism were unreliable because of the low prevalence. Finally, data on iodine excretion were not available for all women. Nonetheless, iodine excretion analyses in a random sample of 1099 pregnant women did not show any correlations with premature delivery.

In conclusion, especially women with hypothyroxinemia, but also TPOAb positive women have a substantially increased risk of a premature delivery. Associations between elevated TSH levels and premature delivery were not seen in spontaneous premature deliveries and depended on the concomitant occurrence of TPOAb positivity and/or comorbidities. Finally, we show that the association between TPOAb positivity and premature delivery is independent of thyroid function. These data give insight into the effects of abnormal thyroid function during early pregnancy and the risk of a premature delivery and suggest that screening for TPOAb positivity and hypothyroxinemia could be considered, especially among women with other risk factors for premature delivery.

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CHAPTER 12

THE ASSOCIATION OF MATERNAL THYROID FUNCTION DURING EARLY PREGNANCY WITH OFFSPRING IQ AND BRAIN MORPHOLOGY AT SCHOOL-AGE: A POPULATION-BASED PROSPECTIVE COHORT STUDY

Tim I.M. Korevaar, Ryan Muetzel, Marco Medici, Layal Chaker, Vincent W.V. Jaddoe, Yolanda B. de Rijke, Eric A.P. Steegers, Theo J. Visser, Tonya White, Henning Tiemeier and Robin P. Peeters

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ABSTRACT

BACKGROUND Thyroid hormone is involved in the regulation of early brain development. Since the fetal thyroid gland is not fully functional until week 18–20 of pregnancy, neuronal migration and other crucial early stages of intrauterine brain development largely depend on the supply of maternal thyroid hormone. Current clinical practice mostly focuses on preventing the negative consequences of low thyroid hormone concentrations, but data from animal studies have shown that both low and high concentrations of thyroid hormone have negative effects on offspring brain development. We aimed to investigate the association of maternal thyroid function with child intelligence quotient (IQ) and brain morphology.

METHODS In this population-based prospective cohort study, embedded within the Generation R Study (Rotterdam, Netherlands), we investigated the association of maternal thyroid function with child IQ (assessed by non-verbal intelligence tests) and brain morphology (assessed on brain MRI scans). Eligible women were those living in the study area at their delivery date, which had to be between April 1, 2002, and Jan 1, 2006. For this study, women with available serum samples who presented in early pregnancy (<18 weeks) were included. Data for maternal thyroid-stimulating hormone, free thyroxine, thyroid peroxidase antibodies (at weeks 9–18 of pregnancy), and child IQ (assessed at a median of 6.0 years of age [95% range 5.6–7.9 years]) or brain MRI scans (done at a median of 8.0 years of age [6.2–10.0]) were obtained. Analyses were adjusted for potential confounders including concentrations of human chorionic gonadotropin and child thyroid-stimulating hormone and free thyroxine.

FINDINGS Data for child IQ were available for 3839 mother–child pairs, and MRI scans were available from 646 children. Maternal free thyroxine concentrations showed an inverted U-shaped association with child IQ (p=0.0044), child grey matter volume (p=0.0062), and cortex volume (p=0.0011). For both low and high maternal free thyroxine concentrations, this association corresponded to a 1.4–3.8 points reduction in mean child IQ. Maternal thyroid-stimulating hormone was not associated with child IQ or brain morphology. All associations remained similar after the exclusion of women with overt hypothyroidism and overt hyperthyroidism, and after adjustment for concentrations of human chorionic gonadotropin, child thyroid-stimulating hormone and free thyroxine or thyroid peroxidase antibodies (continuous or positivity).

INTERPRETATION Both low and high maternal free thyroxine concentrations during pregnancy were associated with lower child IQ and lower grey matter and cortex volume. The association between high maternal free thyroxine and low child IQ suggests that levothyroxine therapy during pregnancy, which is often initiated in women with subclinical hypothyroidism during pregnancy, might carry the potential risk of adverse child neurodevelopment outcomes when the aim of treatment is to achieve high-normal thyroid function test results.

INTRODUCTION

Mild maternal thyroid hormone deficiency occurs in about 5–18% of all pregnant women worldwide, depending on the definition used.[1-4] Thyroid hormone is crucial for intrauterine neurodevelopment because it regulates migration, proliferation, and differentiation of fetal neuronal cells that form grey matter later in life, as well as synaptogenesis and myelination.[5,6] In human beings, early neurogenesis starts from approximately 5 weeks post-conception and thyroid hormone receptors have been detected in fetal brain from as early as 8 weeks.[7] Since the fetal thyroid gland is not functionally matured before week 18–20 of pregnancy,[8] the fetus largely depends on the supply of maternal thyroxine during the early stages of intrauterine brain development.

Results from animal studies have shown that shortage of thyroid hormone impairs brain development and affects brain morphology.[5,6] Brain morphology, particularly relative grey matter volume and cortical thickness, shows a consistent positive association with intelligence quotient (IQ).[9] Data from population studies have shown that the offspring of women with low free thyroxine concentrations during early pregnancy (either classified as overt hypothyroidism or hypothyroxinaemia) have a deficit of up to seven IQ points compared with a control group of offspring from non-hypothyroid women.[10,11] Although thyroid-stimulating hormone is often regarded as the best marker for thyroid function during pregnancy, no properly executed clinical study has shown that a raised concentration of thyroid-stimulating hormone in the presence of a normal free thyroxine is associated with impaired neurocognitive development in the offspring. In line with the general belief that only low maternal free thyroxine concentration during pregnancy is negatively associated with offspring IQ, the association of hypothyroxinaemia (as compared with the rest of the population) with brain morphological outcomes was previously assessed,[12] but the use of this methodology in this study did not yield statistically significant results. Although longstanding evidence from animal studies also suggests that high concentrations of thyroid hormone adversely affect brain development, presumably via a counterproductive acceleration of neuronal cell proliferation, differentiation, and migration,[13-19] the association between high maternal thyroid function and offspring brain development outcomes has not previously been studied in human beings.

Overt gestational hypothyroidism is rare (affecting 0.2–0.5% of pregnant women worldwide) and the use of levothyroxine treatment for this disease entity is undisputed.[1-3] Milder types of thyroid dysfunction (ie, subclinical hypothyroidism) are up to ten-times more prevalent than overt gestational hypothyroidism, and existing treatment recommendations are mainly based on data from observational studies of low maternal thyroid function and clinical outcomes.[1-3] Although only poor-grade to fair-grade evidence exists for the treatment of mild thyroid hormone deficiency during pregnancy,[1-3] women with subclinical hypothyroidism often receive levothyroxine treatment since the potential benefits are believed to outweigh the potential risks of overtreatment.[1,2] However, practically no data are available about the potential harmful effects of high maternal thyroid hormone concentrations on child brain development.

The main aim of this study was to investigate the associations of early pregnancy maternal thyroid function with child IQ (assessed by a non-verbal intelligence test) and brain morphology (assessed by MRI) in a large, population-based, prospective cohort.

METHODS

Study design and participants

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, Netherlands.[20] Eligible mothers were those living in the study area (the municipality of Rotterdam) and had an expected delivery date between April 1, 2002, and Jan 1, 2006,[20] and were enrolled when reporting pregnancy to the midwife or at the hospital. The main exclusion criteria were: abortion, fetal loss, or gestation age at 24 weeks or more at inclusion. For this study, women were eligible when enrolled during early pregnancy (<18 weeks' gestation) and when blood samples were taken to enable measurement of thyroid-stimulating hormone, free thyroxine, and antibodies for thyroid peroxidase. When the children reached 5 years of age, all enrolled mothers and children were invited to visit the research centre at the Erasmus MC Sophia Children's Hospital in Rotterdam, where the children underwent IQ assessments. A subgroup of children, selected partly at random,[21] were subsequently invited to undergo brain MRI brain scanning, also at the Erasmus MC Sophia Children's Hospital, between Sept 1, 2009, and Feb 28, 2012. Missing outcome data were mainly due to non-differential non-response to invitation.

The general study design, all research aims, and the specific measurements in the Generation R Study have been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, Netherlands. Written informed consent was obtained from all participants and/or the childrens' parents or guardians.

Procedures

Maternal serum samples were obtained in early pregnancy, cord blood samples were obtained directly after birth, and child serum samples were obtained at the time of IQ measurement. Plain tubes of blood samples were centrifuged and serum was stored at –80°C. Thyroid-stimulating hormone and free thyroxine concentrations were measured in maternal and cord blood serum samples by use of chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY, USA). Additional information about serum measurements is provided in the appendix. Overt hypothyroidism was defined biochemically as an increased concentration (>97.5th population percentile) of thyroid-stimulating hormone with a decreased concentration (<2.5th population percentile) of free thyroxine, and overt hyperthyroidism was defined as decreased concentration of thyroid-stimulating hormone with increased concentration of free thyroxine, after exclusion of thyroid peroxidase antibody-positive women, as advocated in international guidelines.[1-3]

Non-verbal child IQ was assessed with the use of two subtests of a well-validated Dutch non-verbal intelligence test, the Snijders-Oomen Niet-Verbale Intelligentie Test, when the children were around 6 years of age. This test is regarded as highly reliable and rated good (3 out of 3) by the commission of the Netherlands Insitute for Psychologists.[22] The test broadly assesses a range of intelligence functions without depending on language skills and is therefore appropriate for the assessment of cognitive abilities of children of ethnic minorities and children who have problems with verbal communication. The two subtests were mosaics, which assesses spatial visualisation abilities, and categories, which assesses abstract reasoning abilities (correlation subtests with complete test: r=0.86); raw test scores were converted into non-verbal IQ scores with the use of normal values tailored to exact age. Research staff who did the IQ tests were unaware of any other test outcomes, including maternal thyroid function during pregnancy.

Structural MRI scans were done with a GE Discovery MR750 3.0 Tesla scanner (General Electronics Healthcare, Little Chalfont, Buckinghamshire, UK), using an eight-channel head coil. The high-resolution,

T1-weighted image was obtained via an inversion recovery fast spoiled gradient recalled sequence. MRI outcomes were total grey matter volume, cortex volume, total white matter volume, hippocampal volume, and total brain volume; all outcomes were assessed automatically by computer algorithms with use of the FreeSurfer pipeline. Scan sequence parameters, image quality assurance, and image processing are described in detail in the appendix.

Statistical analyses

Thyroid-stimulating hormone and free thyroxine concentrations were logarithmically transformed. We investigated the shape of the association between thyroid-stimulating hormone or free thyroxine concentrations and child IQ or brain MRI outcomes using ordinary least-squares linear regression models with restricted cubic splines with three knots at the 10th, 50th, and 90th percentiles, and built multiple linear regression models accordingly. Additionally, we also created standard multivariate linear regression models with a quadratic term to provide effect estimates. We investigated the risk of the children having an IQ lower than 85, as a limit for borderline intellectual functioning, using logistic regression models with restricted cubic splines with three knots at the 10th, 50th, and 90th percentiles. Further See Online for appendix details about the statistical analyses are reported in the appendix.

We tested several confounders for each model outcome and we selected or excluded covariates on the basis of biological confounding plausibility or change in effect estimate of the variable of interest, or the reduction of residual variance of the model. Analyses of IQ (both linear and of scores <85) were adjusted for gestational age at blood sampling, maternal age, smoking, BMI, parity, education level, ethnic origin, fetal sex, and birthweight. Analyses of MRI parameters were adjusted for gestational age at blood sampling, maternal age, BMI, child age, sex, birthweight, and gestational age at birth. We also considered breastfeeding and Child Behaviour Checklist/1.5–5 scores (see appendix for details of data ascertainment) as potential confounders, but adjustment for these variables did not change the results and therefore we did not include them in the final models. Additionally, we adjusted all analyses for human chorionic gonadotropin, thyroid peroxidase antibodies, and child thyroid function based on the underlying physiology by sequentially adding these variables to the model. We did not consider adjusting for multiple comparisons in view of the hypothesis-based approach and the low number of tested hypotheses. Further details about the statistical analyses are reported in the appendix. All statistical analyses were done with R statistical software version 3.03 (package rms) or SPSS version 20.0 for Windows.

Role of the funding source

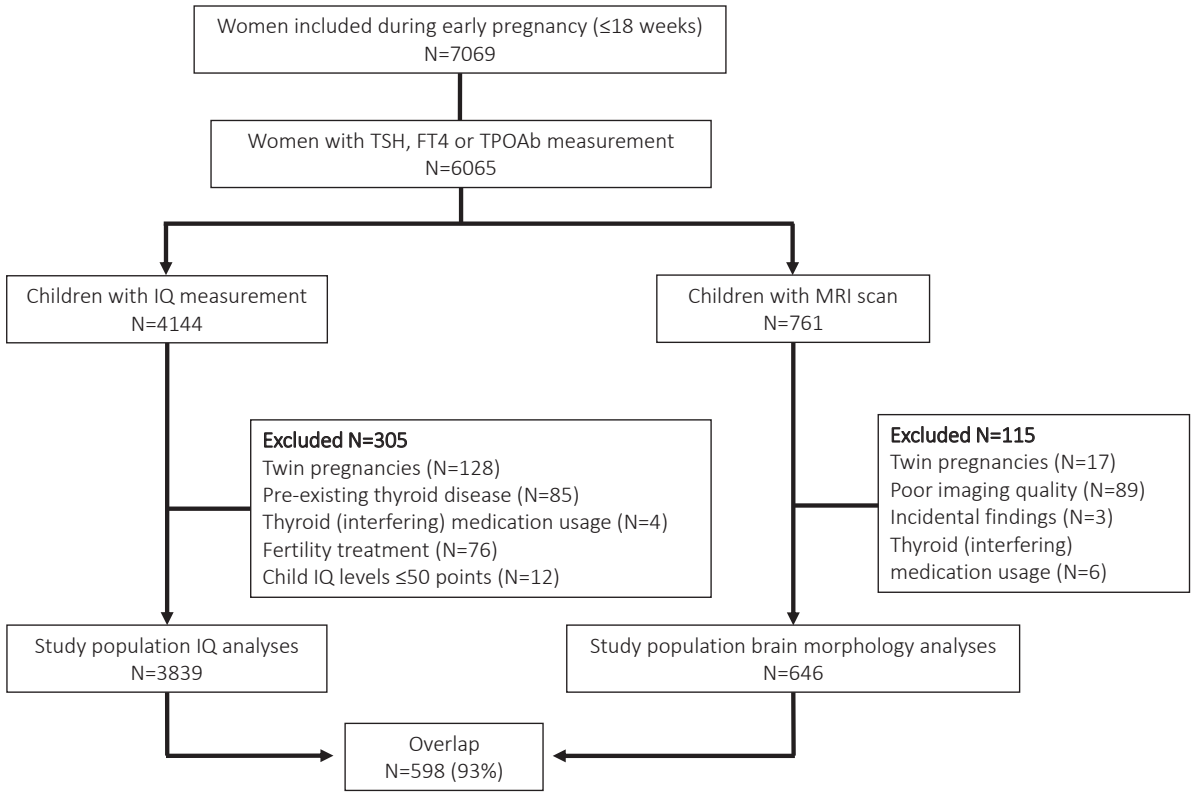
The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

RESULTS

A total of 7069 women were enrolled during early pregnancy, and blood samples were available from 6398. Measurements of thyroid-stimulating hormone, free thyroxine, or thyroid peroxidase antibody were obtained for 6065 mothers; median gestational age at serum measurements was 13.2 weeks (95% range 9.8–17.5). Cord blood samples were obtained directly after birth (median gestational age at birth 40.1 weeks [95% range 35.9–42.3]). IQ was assessed in 4144 children at a median age of 6.0 years (95% range 5.6–7.9 years). A subgroup of 1252 children were invited to undergo brain MRI scans, of whom

801 children agreed and scans were done at a median age of 8.0 years (95% range 6.2–10.0). Of these 801 children, data for maternal thyroid-stimulating hormone, free thyroxine, or thyroid peroxidase antibody concentrations during pregnancy were available for 761. After exclusions, 3839 mother– child pairs were included in the study population for IQ analyses and 646 mother–child pairs were included in the study population for brain morphology; IQ and MRI data overlapped for 598 mother–child pairs (figure 1).

FIGURE 1. *Flowchart for mother-childpairs in the study.*



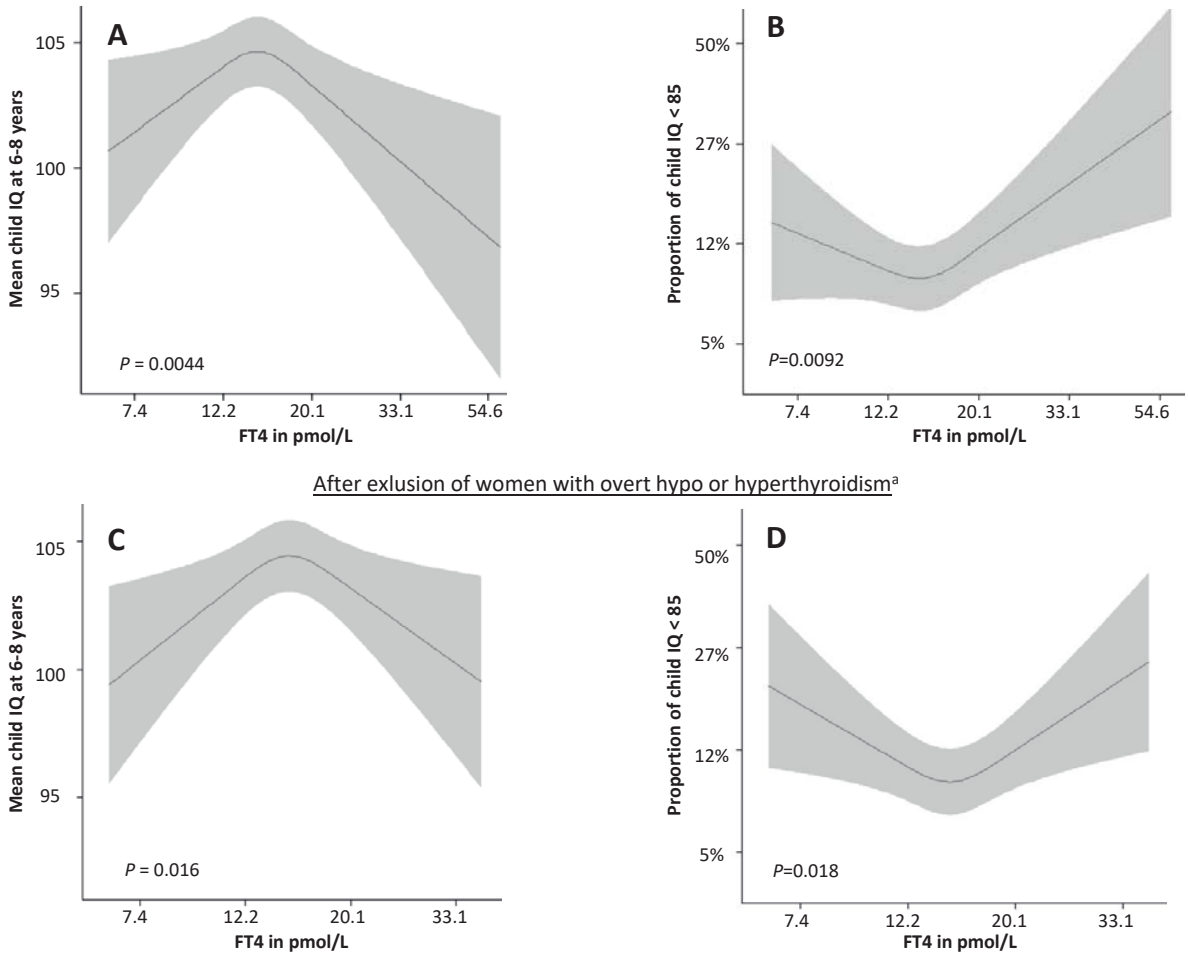
Maternal thyroid-stimulating hormone concentrations, free thyroxine concentrations, and thyroid peroxidase antibody positivity did not differ between mothers who had a child with IQ data available and those who did not (data not shown; differences for child thyroid function are described in the appendix). Similarly, there were no differences in maternal thyroid-stimulating hormone or free thyroxine concentrations, or thyroid peroxidase antibody positivity, or a difference in child non-verbal IQ, for children with MRI data available versus those without MRI data (data not shown). Maternal thyroid-stimulating hormone concentration, free thyroxine concentration, or thyroid peroxidase antibody positivity also did not differ between groups on the basis of data availability (ie, only IQ data vs only MRI data vs both IQ and MRI data; data not shown). Median maternal thyroid-stimulating hormone con centration was 1.35 mU/L (normal range 0.03–4.04 mU/L) and median maternal free thyroxine concentration was 14.9 pmol/L (normal range 10.4-22.0 pmol/L). The study population was mainly of Dutch origin (table 1). Mean child IQ was 101.5 points (SD 14.9), and 515 (13%) of 3839 children had an IQ lower than 85 (table 1). Descriptive statistics for women with high free thyroxine are in shown in table s1.

TABLE 1 *Descriptive statistics of 3839 mother-child pairs in the study population.*

		Median	(95% range)
TSH	(mU/L)	1.35	(0.05 - 4.96)
FT4	(pmol/L)	14.9	(10.2 - 22.4)
hCG	(IU/L)	44,664	(12,472 – 105,829)
Iodine to creatinine ratio*	(µg/g)	227	(97-703)
TPOAb positivity		5.5%	
Gestational age[a]		13.2	(9.8 - 17.5)**
Maternal aged		30.9	(20.1 - 39.1)
BMI		23.5	(18.7 - 35.4)
Parity[c]			
0		2279	(59.4)
1		1128	(29.4)
2		320	(8.3)
>2		112	(2.9)
Smoking[c,e]			
Non-smokers		2874	(74.9)
Stopped smokers		381	(9.9)
Smokers		584	(15.2)
Education level[e]			
None/Primary		292	(7.6)
Secondary phase 1		487	(12.7)
Secondary phase 2		1203	(31.3)
Higher phase 2		884	(23.0)
Higher phase 1		974	(25.3)
Ethnicity[c,e]			
Dutch		2169	(56.5)
Moroccan		198	(5.2)
Turkish		266	(6.9)
Surinamese		294	(7.7)
Cape Verdian		159	(4.1)
Dutch Antilles		72	(1.9)
Indonesian		119	(3.1)
Asian		87	(2.3)
Other western		329	(8.6)
Other non-western		146	(3.8)
Child IQ (mean(SD))		101.5	(14.9)
Child IQ < 85		13.4%	
Child TSH	(mU/L)	2.31	(0.88 - 5.14)
Child FT4	(pmol/L)	16.7	(13.7 - 20.6)
Child TSH at birth	(mU/L)	9.65	(3.41 – 34.8)
Child FT4 at birth	(pmol/L)	20.5	(15.4 – 28.3)
Birth weight (g)		3450	(2240 – 4450)
Child gender[c] (boys %)		1886	(49.1)

** In random subset of urine samples from N=672 women*
*** Full range = 6-18 weeks.*
[a] *At time of blood sampling; data shown as median in weeks*
[b] *Data shown as mean in (SD)*
[c] *Data shown as n (%)*
[d] *Data shown as median in years*
[e] *Data shown after imputation of missing data (see methods).*

FIGURE 2. *The association of maternal FT4 with offspring IQ.*

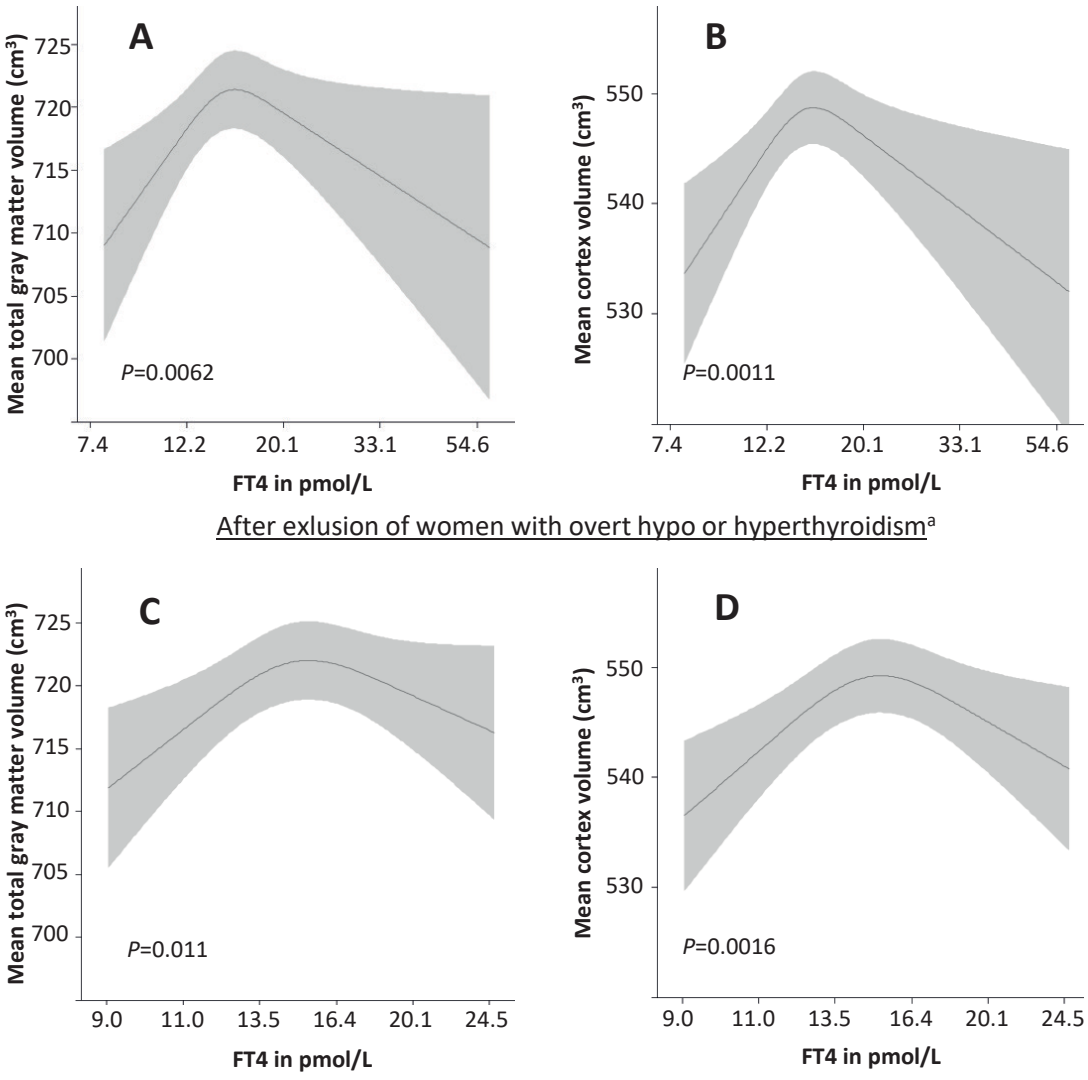


[a] Overt hypo and hyperthyroidism is defined as the biochemical diagnosis made during pregnancy based on the central 95% reference range as advocated by international guidelines. Plots A and C show the association between maternal FT4 levels during pregnancy and child IQ as predicted mean with 95 percent confidence interval in the whole population (A) and after exclusion of women with overt thyroid disease (C); plots B and D show the association of maternal FT4 levels during pregnancy and the risk of child IQ <85 as predicted, backtransformed log odds with 95 percent confidence interval in the whole group (B) and after exclusion of women with overt disease (D). All analyses (A-D) were performed with R statistical package using the RMS package amongst singleton pregnancies after exclusion of women with IVF treatment (N=76) or women with known thyroid disorders or thyroid interfering medication usage (N=89) and were adjusted for gestational age at blood sampling, hCG, maternal age, smoking, BMI, parity, education level, ethnicity, fetal gender and birth weight. Scales for the x-axis may differ between upper and lower graphs due to exclusions of women with overt, but not subclinica, disease entities. See Table S2 for effect estimates of standard linear regression models.

Our results show an inverted U-shaped association of maternal free thyroxine concentration with child IQ (p=0.0044, figure 2A; results from standard linear regression models are shown in table s2). The association between maternal free thyroxine and child IQ remained similar after the exclusion of women with a clear treatment indication based on international guidelines (ie, overt hypothyroidism [n=15] and overt hyperthyroidism [n=34]; figure2C). Maternal thyroid-stimulating hormone concentrations were not associated with child IQ (figure s1; p=0.05) and when both maternal thyroid-stimulating hormone and free thyroxine concentrations were added to the model, the association of maternal thyroid-stimulating hormone with offspring IQ attenuated (p=0.36), whereas the association for free maternal thyroxine remained similar (p=0.03). Secondary analyses for different percentile cutoff levels showed that high maternal free thyroxine concentration was associated with a statistically significant 1.4–3.7

points reduction in mean child IQ (range of cutoff levels: >88th to >97th percentile, all p≤0.05; appendix (0.05; figure S2)). Low maternal free thyroxine concentration was associated with a statistically significant 1.5–3.8 points reduction in mean child IQ (range of cutoff levels: <3rd to <11th percentile, all p≤0.05; appendix (0.05; figure S2)). We also noted a U-shaped association between maternal free thyroxine concentration and child IQ lower than 85 points, and this finding remained similar after exclusion of women with a treatment indication based on international guidelines (figure 2B, 2D).

FIGURE 3. *The association between maternal FT4 and offspring brain morphology.*



[a] Overt hypo and hyperthyroidism is defined as the biochemical diagnosis made during pregnancy based on the central 95% referencerange as advocated by international guidelines.Plots show the association between maternal FT4 levels during early pregnancy and child MRI brain morphology outcomes as predicted mean with 95 percent confidence interval. Analyses were performed with R statistical package using the RMS package amongst singleton pregnancies and were adjusted for gestational age at blood sampling, maternal age, BMI, child age, gender, birth weight, gestational age at birth and total brain volume. Scales for the x-axis may differ between upper and lower graphs due to exclusions of women with overt, but not subclinica, disease entities. See Table S3 for effect estimates of standard linear regression models.

The associations between maternal thyroid-stimulating hormone or free thyroxine concentrations and child IQ did not differ on the basis of gestational age at time of blood sampling (product interaction

term for thyroid-stimulating hormone or free thyroxine and gestational age at blood sampling: p=0.28 and p=0.88, respectively). The association between maternal free thyroxine concentration and child IQ remained essentially unchanged after correction for maternal human chorionic gonadotropin, thyroid peroxidase antibodies (continuous or positivity), and child thyroid-stimulating hormone and free thyroxine concentrations (at birth or at time of IQ measurement; data not shown). When only the 3602 women with free thyroxine concentrations within the gestational reference range were included in the analysis, the overall association of maternal free thyroxine concentration during pregnancy with mean child IQ did not remain statistically significant (p=0.26; figure s3), whereas the association with the risk of child IQ lower than 85 did remain statistically significant (p=0.041; figure s3). Total grey matter volume and cortex volume were positively associated with child IQ (figure s4). Similar to the results for child IQ, we recorded an inverted U-shaped association of maternal free thyroxine concentration with child total grey matter (p=0.0062), and cortex volume (p=0.0011), which remained similar after the exclusion of women with overt hypothyroidism and overt hyperthyroidism (figure 3A–D, appendix).

Maternal free thyroxine concentration was not associated with total brain volume, white matter volume, hippocampal volume (table s3), or subcortical grey matter volume (data not shown). Maternal thyroid-stimulating hormone concentration was associated with child total grey matter volume and cortex volume, but these associations disappeared after correction for total brain volume (figure s1). All results were independent of maternal human chorionic gonadotropin, thyroid peroxidase antibodies, or child thyroid-stimulating hormone and free thyroxine concentrations (data not shown). Within gestational normal-range free thyroxine concentrations, the overall association of maternal free thyroxine concentration during pregnancy with mean child grey matter or cortex volume did not remain statistically significant (n=613; p=0.09 and p=0.07, respectively [figure s3]). Within the group of 598 children with both IQ and MRI measurements, the association between maternal free thyroxine concentration and child IQ was not statistically significant (mediation analysis not possible; data not shown).

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DISCUSSION

In this large, population-based, prospective cohort study, we investigated the association of maternal thyroid function during early pregnancy with child IQ and corresponding changes in brain morphology. To our knowledge, this is the first study to show that high maternal free thyroxine concentrations are associated with lower child IQ, which, together with a similar effect for low maternal free thyroxine concentrations, results in an overall inverted U-shaped association. The association of maternal free thyroxine concentrations with child IQ was consistent with novel neuroimaging data, which showed that offspring from mothers with both high and low maternal free thyroxine concentrations had reduced total grey matter and lower cortex volumes. In line with our analyses on child IQ, our results showed an inverted U-shaped association for the full range of maternal free thyroxine concentrations with child total grey matter volume and cortex volume. These associations were independent of differences in maternal human chorionic gonadotropin, thyroid peroxidase antibodies, or child thyroid-stimulating hormone and free thyroxine concentrations.

Child IQ is associated with many health outcomes and lifetime achievements (including educational achievement, well-paid employment, enhanced social status, and the accompanying benefits to health): every IQ point lost from the US average is estimated to have an annual cost of US$71 billion.[23,24] To our knowledge, our study is the first in human beings to show a negative association between high maternal free thyroxine concentrations during pregnancy and early brain development in children.

Our observations are consistent with the longstanding results from studies in animals showing that hyperthyroidism accelerates different postnatal brain development processes, leading to suboptimal brain development through decreased synaptogenesis, myelination, cortical growth, and a reduced number of astrocytes.[13-15] Although these studies of postnatal brain development in rats resemble human third trimester brain development, which does not relate exactly to our present study in which thyroid function was measured during early pregnancy, such findings illustrates the negatives consequences of high levels of thyroid hormone for brain development. In the cerebellum, high postnatal doses of thyroid hormone in rats result in premature differentiation and migration of granule cells, decreased foliation, and insufficient arborisation of Purkinje cells.[16,18,25] Finally, the high activities of the thyroid hormone-inactivating enzyme, type 3 deiodinase (D3) in various regions of the human fetal brain also suggest a need to limit exposure to thyroid hormone during immature developmental stages.[26,27]

In addition to the brain, the placenta is also a site of high D3 activity, suggesting an additional role of D3 in protecting the fetus by preventing excessive maternal thyroid hormone transfer.[28] This idea is substantiated by placental perfusion studies showing an approximately 2700-times increase in concentrations of free thyroxine at the fetal site after D3 inhibition.[28] Comparing the effects of prenatal thyroid hormone exposure on offspring brain development from rat studies with the current study, our results suggest a smaller effect in human beings than in rats, which is probably attributable to the presence of the placental barrier in human beings. However, the fetus might be most vulnerable to high maternal thyroid hormone concentrations during early pregnancy because during this period the placental surface area is still relatively small and D3 activity is low.[29] The hypothesis that placental D3 activity can be saturated[30] and might not be able to fully protect the fetus from high maternal thyroid hormone concentrations during early pregnancy is supported by clinical data showing negative effects of high-normal free thyroxine concentrations on birthweight and fetal loss.[31-33]

Previous studies of the effects of maternal thyroid hormone concentrations on various adverse clinical outcomes, including child IQ, laid the foundation for treatment recommendations in current international guidelines.[1-3,10,11] Treatment of subclinical hypothyroidism ie, high thyroid-stimulating hormone and normal free thyroxine concentrations—and of a thyroid-stimulating hormone concentration higher than 10 mU/L is recommended in the guidelines of the American Thyroid Association[3] (subclinical hypothyroidism in case of thyroid peroxidase antibody positivity), the Endocrine Society,[1] and the European Thyroid Association[2] to prevent the negative consequences of low thyroid hormone concentrations during early pregnancy on various pregnancy outcomes and offspring development. The potential benefits of treatment are generally believed to outweigh the potential risks[2,3], and in clinical practice the treatment aim is often set to the high-normal thyroid function range to quickly overcome insufficient thyroid hormone concentrations. The association of maternal free thyroxine with child IQ and corresponding changes in brain morphology in the present study is driven mainly by the effects of both low and high free thyroxine concentrations. However, when women with a clear treatment indication (ie, overt hypothyroidism or hyperthyroidism) were excluded, the inverted U shape persisted, suggesting that free thyroxine concentrations within the subclinical range also have an important effect on the neurocognitive development of the offspring. Although we did not study women who underwent treatment, our results could suggest that treatment aiming for a high-normal free thyroxine concentration during pregnancy might carry the risk of adverse child neurodevelopment outcomes.

We speculate that the potential risk of overtreatment is especially high in women with gestational subclinical hypothyroidism, because treatment of subclinical hypothyroidism could lead to normalisation of thyroid-stimulating hormone concentrations but high-normal free thyroxine concentrations, since free thyroxine concentrations were normal before the onset of treatment. As part of the Controlled Antenatal

Thyroid Screening study,[4] 10% of the 390 women with subclinical hypothyroidism or hypothyroxinaemia who received a fairly high dose of 150 µg/L free thyroxine treatment during pregnancy needed a dose reduction because of biochemical or clinical signs of overtreatment. We postulate that the risk of overtreatment for women with mild thyroid dysfunction is especially high because the thyroid gland still has residual activity that is stimulated by human chorionic gonadotropin, whereas there is no negative feedback mechanism between free thyroxine and human chorionic gonadotropin. This combination might be prone to result in (transient) raised concentrations of free thyroxine.

Neurocognitive development of the offspring is regarded as a major adverse outcome of maternal thyroid dysfunction during pregnancy, although consistent associations with this outcome have only been shown for maternal free thyroxine, as opposed to maternal thyroid-stimulating hormone.10 Nevertheless, international guidelines recommend treatment aims based solely on thyroid-stimulating hormone concentrations in women with pre-existing thyroid disease or gestational thyroid dysfunction.[1-3] Based on our results showing that the association between maternal thyroid-stimulating hormone and child IQ was weak and merely reflected the changes in maternal free thyroxine concentrations, this could be a suboptimal approach. Data from other large population-based studies are needed to investigate the value of (non-parametric) free thyroxine treatment targets.

To the best of our knowledge, our study is the first to show that maternal thyroid function during pregnancy is associated with offspring brain MRI outcomes in childhood. Relative differences in grey matter volume might indicate differences in the efficiency of information processing.[9] In line with this idea, relative grey matter volume and cortical thickness are positively associated with IQ.[9] We have shown that an association exists between maternal free thyroxine concentration and child total grey matter volume and cortex volume. This finding is in agreement with results of animal studies showing widespread effects of thyroid hormones on neuronal cells during early brain development, and the association of maternal free thyroxine concentration with cortex volume but not subcortical grey matter in particular fits with the effects that have been shown on early neuronal cell migration.[6] Consistent with the analysis of IQ, we identified an inverse U-shaped association of maternal free thyroxine concentration with child grey matter and cortex volume. However, in the much smaller subset of 598 mother–child pairs with data for both IQ and brain MRI, maternal free thyroxine concentration was not associated with child IQ (probably because the sample size was too small to detect an association) and we were therefore unable to investigate whether or not the grey matter changes mediate the results for IQ. Future studies are therefore needed to investigate potential mediation and whether the effects of maternal thyroid function on child brain function and morphology are area specific.

Two small-scale studies by Willoughby and colleagues[34] and Samadi and colleagues[35] showed that children born from mothers with overt hypothyroidism during pregnancy had smaller hippocampal volume and abnormal corpus callosum measurements than did controls. In our study, we did not identify an association between maternal thyroid function and hippocampal or corpus callosum volume. This discrepancy is most likely explained by the fact that the other two studies included women who had either overt hypothyroidism throughout pregnancy or untreated overt hypothyroidism during at least the first trimester.[34,35] Further studies are needed to investigate the time-dependent effects of maternal thyroid function on child functional and structural brain outcomes.

Our study investigated the association between the full range of maternal thyroid function and both child IQ and brain morphology. Previous studies defined low maternal thyroid function by different thyroid-stimulating hormone or free thyroxine cutoffs, and compared such groups with the remainder of the population or matched controls. In the same population as this study, a similar cutoff-based approach yielded negative results because non-linear associations were not investigated.[12] Although the hypotheses of clinical studies investigating the association of maternal thyroid hormone concentrations

and offspring brain development are based on a wide range of animal studies, it is important to note that observational studies can still be vulnerable to the effects of residual confounding and therefore cannot infer causality.

Previous studies that investigated the association between low maternal thyroid function during pregnancy and child neurocognitive functioning did not adjust for human chorionic gonadotropin, thyroid peroxidase antibody status, or childhood thyroid function, but these factors did not affect the associations identified in this study. A potential limitation of our study is that only one measurement of maternal thyroid function was available. In theory, the low or high free thyroxine concentrations during early pregnancy measured in this study could have been transient, not representing thyroid hormone availability during the full course of pregnancy. However, a high correlation between longitudinal thyroid function measurements has previously been shown[36-38] and we also noted that the association between maternal thyroid-stimulating hormone or free thyroxine concentrations and child IQ were similar throughout the gestational period in which this study was done.

The fact that all widely used free thyroxine assays are affected by the pregnancy-specific increase in binding proteins could also limit the interpretation of our results. However, this increase in proteins mainly occurs in the third trimester of pregnancy, whereas our study was done in the first half of pregnancy. Additionally, we used a chemiluminescence assay that has a low bias and high concordance compared with direct equilibrium dialysis in sera from patients with different states of high thyroxine-binding capacities, including pregnancy, which suggests that, although the absolute values might differ, high correlation exists between values.[38] Finally, it is very unlikely that such assay variability caused biased effect estimates on child IQ, grey matter volume, or cortex volume, since it is very unlikely that free thyroxine assay variability is differential on offspring outcomes.

Another potential limitation of our study is the fact that we only had iodine data available from a small subset of 672 women (table 1), and iodine status could adversely affect both thyroid function and child brain development.[39] Although this study was undertaken in a population with proven iodine sufficiency, we should note that iodine-deficient women, and those with high iodine intake, could still be present in the study population.[40] For women with an insufficient or excessive iodine intake, we would expect to see a decrease in thyroid function,[41] which therefore would only affect the association of low (and not high) maternal free thyroxine concentrations with child IQ and grey matter volume in our study. Although the subset of women with urinary iodine, creatinine, and IQ or MRI measurements available was too small for reliable analyses to be undertaken, a previous study done in this cohort did not show an association between iodine status and child IQ.[40]

In conclusion, both low and high free thyroxine concentrations are associated with a statistically significant decrease in child IQ and also negatively affects child grey matter volume as assessed by MRI. These data suggest that a treatment approach aiming for high-normal thyroid function test results to ensure adequate treatment might come with the potential risk of adverse child neurodevelopment outcomes. More studies are needed to replicate these findings and to further define optimum thyroid status during pregnancy.

SUPPLEMENTS

Methods

In a subset of 2397 children cord blood TSH and FT4 were available, and in 2460 children TSH and FT4 levels were available at time of IQ testing. A comparison between the children who had cord blood, or late thyroid function available and those who had missing data showed no differences between

maternal TSH, FT4 or TPOAb positivity. However, there was a difference in child IQ between the groups (mean increase of 1.3 and 1.0 points for cord blood and late thyroid function, respectively). Children with an IQ ≤ 50 were excluded because the IQ test outcome does not go below 50 and these children scored poorly on motivation, concentration, collaboration and/or understanding of instructions.

Serum measurements

Between April 2002 and January 2006, serum samples were obtained from 6398 women during early pregnancy. Due to measurement error (laboratory) , TSH, FT4 or TPOAb were obtained in 6065 mothers. The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L (for Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/ml. Maternal total human chorionic gonadotropin (hCG) levels (same sample as thyroid function) were analyzed in serum using an Immulite XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA), details of which have been described previously.[42] Child TSH and FT4 levels at time of IQ measurement were determined using an electrochemiluminescence immunoassay on the Cobas e601immunoanayzer (Roche Diagnostics, Germany). The intra- and interassay coefficients of variation were 1.1 – 3.0 % for TSH at a range of 0.4 – 0.04 mU/L and 1.6 – 5.0 % for FT4 at a range of 1.6 -24.1 pmol/L. Corresponding maternal FT4 MoM values [43] for the 1st – 10th percentile were 0.64, 0.67, 0.70, 0.73, 0.74, 0.75, 0.76, 0.77, 0.79 and 0.80 respectively. For the 90th – 99th percentile MoM values were 1,28, 1.31, 1.33, 1.35, 1.38, 1.40, 1.45, 1.49, 1.56 and 1.70, respectively.

Determinants and covariates

Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[44] Information on maternal age, smoking status, education level and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands.[20] Maternal ethnicity was categorized according to the major ethnic groups in Rotterdam, the Netherlands (Dutch, Moroccan, Turkish, Surinamese, Indonesian, Cape Verdian or Antillean) and the remaining women were grouped into Asians, other Western or other non-Western. Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. Maternal education level consisted of five categories: no education finished/primary school, secondary phase one, secondary phase two, higher education phase one, higher education phase two. Weight and length were measured at intake and were used to calculate body mass index (BMI). Information on fertility treatment, delivery, pregnancy outcome, date of birth, birth anthropometrics, and the gender of the child were obtained from community midwives, obstetricians, and hospital registries. Medical and obstetrical history were assessed by questionnaires and answers were crosschecked by certified medical doctors. Information about initiation of breastfeeding was collected from delivery reports; data on continuation of breastfeeding was obtained from postal maternal self-report questionnaires at 2, 6 and 12 months postpartum, details have been described elsewhere.[45] Child behavior checklist/1½–5 (CBCL/1½–5) was used to obtain a standardized parental rating of child's emotional and behavioral problems. The CBCL/1½–5 contains 99 problem items, scored on seven syndromes that were derived by factor analyses: Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behavior.[46]

MRI data

Exclusion of women with fertility treatment (N=4) did not change the results for total gray matter volume or cortex volume. An overview of the neuroimaging component, including participant selection, has been described elsewhere.[21] An inversion recovery fast spoiled gradient recalled sequence was performed with the following parameters: repetition time = 10.3 msec, echo time = 4.2 msec, inversion time = 350 msec, number of excitations = 1, flip angle = 16, matrix 256 × 256, ASSET factor of 2, and an isotropic resolution of 0.9 × 0.9 × 0.9 mm^3. Before scanning took place, children were familiarized with the scanning environment during a mock scanning session. Volume outcomes are given in $cm^{3.}$

Image quality assurance was performed in two steps. The first step was a visual inspection of the image quality of the T1 sequence prior to preprocessing the data. All images were rated on a six-point scale (unusable to excellent). Research staff that performed MRI ratings are unaware of any test outcomes, including maternal thyroid function during pregnancy. The next step of quality assurance took place after the images were processed through the FreeSurfer pipeline[47] (http://surfer.nmr.mgh.harvard.edu/) and consisted of a visual inspection of the segmentation quality and surface reconstruction of the data. All images were rated on a six-point scale (from poor to excellent). The T1 data that were rated as unusable or poor were not used, nor were the data from the children whose FreeSurfer output was not constructed or were rated as poor for both hemispheres. All outcomes were measured using the FreeSurfer software. The corpus callosum was also automatically segmented and quantified into a volume by summing its voxels in the 5 midline slices, additional quality checks were made by two independent staff members to be certain of data validity for the corpus callosum measurements but an additional stringent quality assessment did not change the results (data not shown).

Image processing

Cortical reconstruction and volumetric segmentation were performed with the FreeSurfer image analysis suite, which has been described in detail elsewhere.[47] Cortical gray matter volume is extracted from the surface reconstructions of both the left and right hemispheres. Total gray matter volume consists of the surface-based measures of cortical gray matter volume described above, plus the volume-based measures of all subcortical structures and cerebellar gray matter. FreeSurfer morphometric procedures have been demonstrated to show good test-retest reliability across scanner manufacturers and across field strengths.[48]

Novel potential confounders

All analyses were additionally adjusted for hCG, TPOAbs and child thyroid function based on the physiologic background. hCG plays an important role in fetal and placental development and may thus affect brain development, while it is also a well-known stimulator of maternal thyroid function during pregnancy. A few small studies reported an association between maternal thyroperoxidase antibody (TPOAb) status and child neurocognitive development but it is unknown if these effects are replicable and whether this is due to an effect on thyroid function or due to the autoimmunity itself.[49-51] Third, maternal hypothyroxinemia may be associated with lower TH levels during childhood and the chronic exposure to subnormal TH levels may influence postnatal brain development and subsequently child IQ levels.[52]

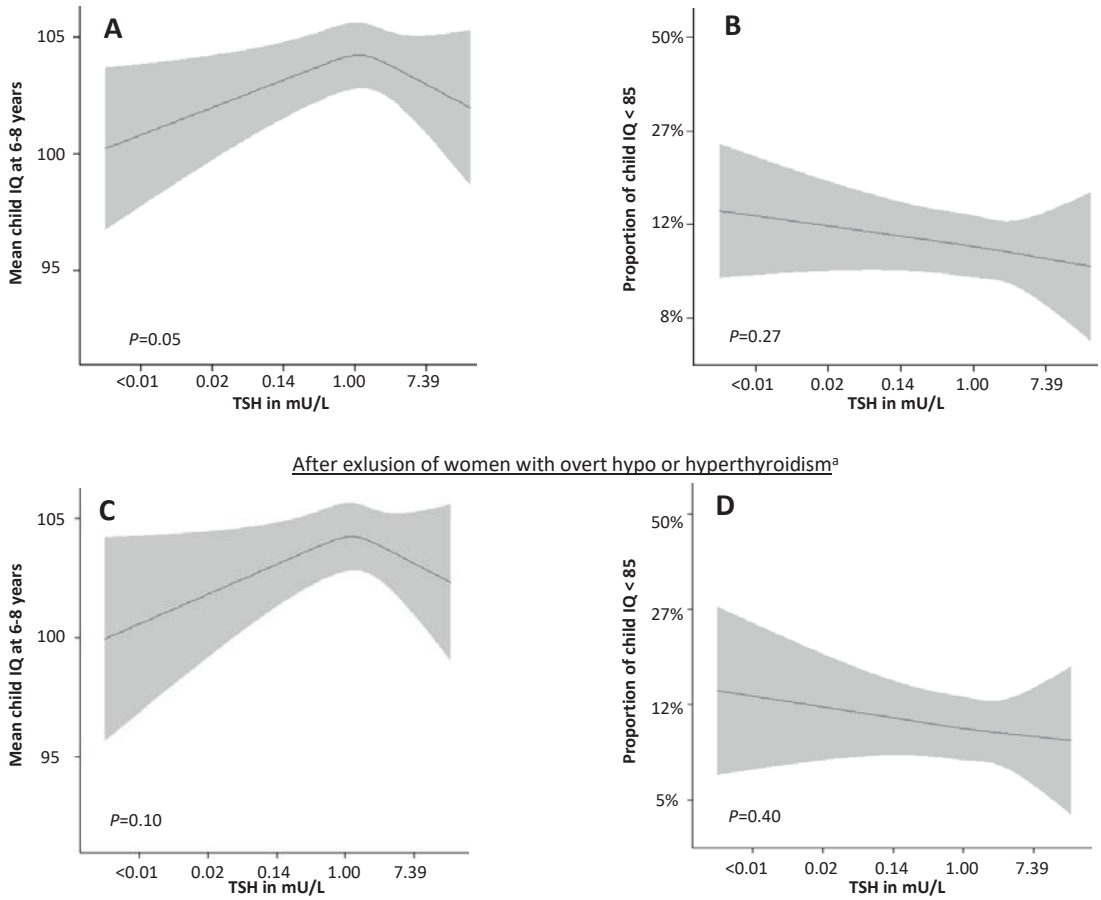
Possible confounding effects of hCG, TPOAbs or child thyroid function were analyzed by additionally adjusting for hCG levels, TPOAbs (continuous or positivity) or child TSH or FT4 levels (at birth and at time of IQ measurement). Additionally, we investigated possible mediation or confounding by child head size (at birth and at IQ measurement), placental factors (including placental blood flow and placental angiogenic factors) and blood flow in the fetal brain (at approximately 25 weeks) for all significant associations but this did not change the results.

Statistical analyses

Figures show back-transformed and/or adjusted axis values (covariates set to mean levels or most appearing category). To investigate if the trend between maternal TSH and mean IQ levels is an independent effect, maternal TSH and FT4 levels were added to the model. We investigated possible differences in gestational time period by adding a product interaction term for TSH or FT4 and gestational age at blood sampling to the model. Cut-off levels for maternal FT4 were investigated using multivariate ANOVA, with <90th percentile or >10th percentile as reference group and cut-off values increasing or decreasing per percentile. For all analyses, model fit and assumptions were assessed by plotting model residuals, evaluating (adjusted) R-squared and/or the le Cessie - van Houwelingen - Copas - Hosmer unweighted sum of squares test. *P*-values shown in figures 2, 3, supplement 1, 3 and 4 test the *null* hypothesis that the mean population IQ is similar for every level of TSH/FT4. For variables with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used (all but TPOAb levels (missing N=243), due to large differences per imputation), five databases were pooled for analyses.

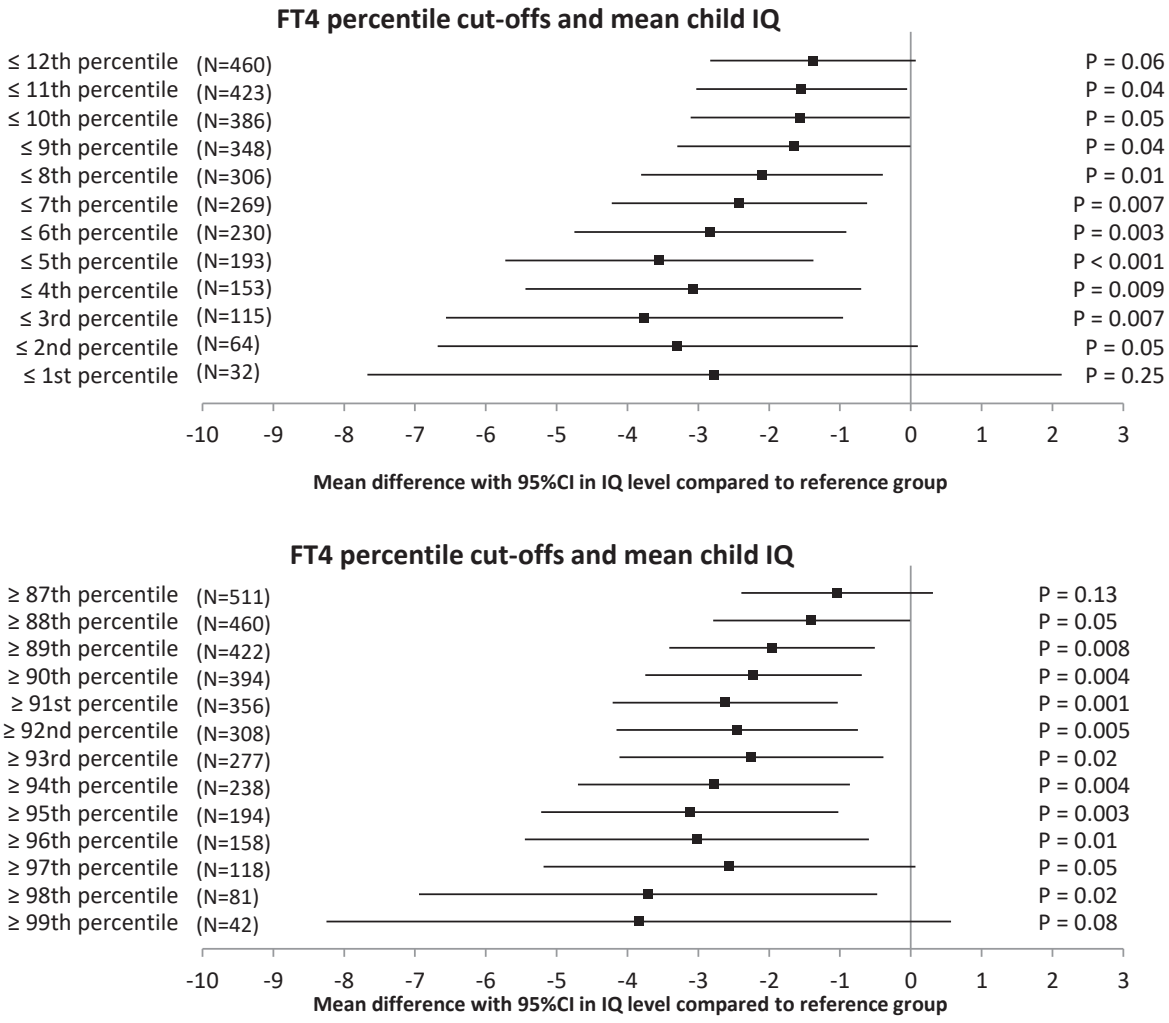
APPENDIX

SUPPLEMENTAL FIGURE 1. *The association between maternal TSH and offspring IQ.*



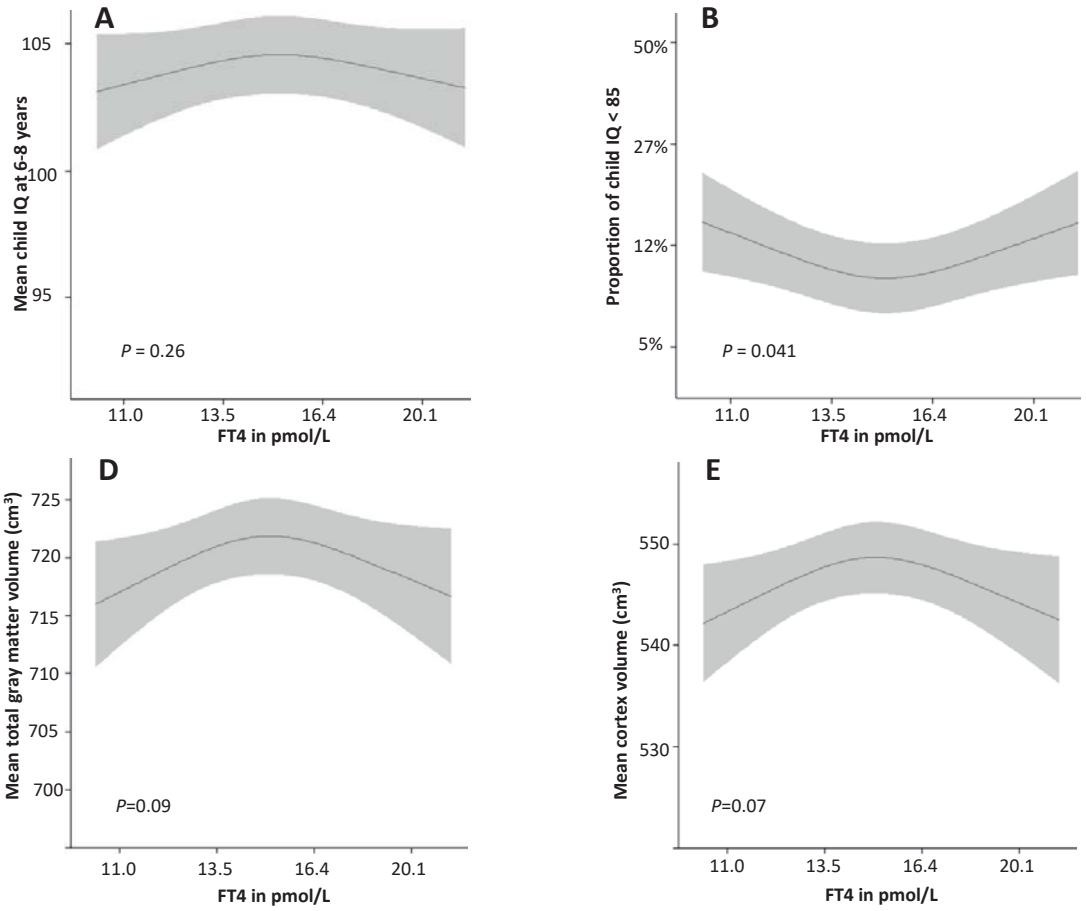
[a] Overt hypo and hyperthyroidism is defined as the biochemical diagnosis made during pregnancy based on the central 95% referencerange as advocated by international guidelines.Plots show the association between maternal TSH levels during pregnancy and child IQ in the whole population (A,B) and after exclusion of women with overt thyroid disease (C,D) as predicted mean with 95 percent confidence interval. Analyses were performed with R statistical package using the RMS package amongst singleton pregnancies after exclusion of women with IVF treatment (N=76) or women with known thyroid disorders or thyroid interfering medication usage (N=89) and were adjusted for gestational age at blood sampling, hCG, maternal age, smoking, BMI, parity, education level, ethnicity, fetalgender and birth weight.See Table S2 for effect estimates of curve linear regression models.

SUPPLEMENTAL FIGURE 2. Sensitivity analyses on cut-off values for low or high FT4 and decreased offspring IQ.



Plots show the mean difference between different percentile-cut-off points for maternal FT4 levels during pregnancy and the reference category of 10th -90thpercentile with 95 percent confidence interval. Analyses were performed amongst singleton pregnancies after exclusion of women with IVF treatment (N=76) or women with known thyroid disorders or thyroid interfering medication usage (N=89) and were adjusted for gestational age at blood sampling, maternal age, smoking, BMI, parity, education level, ethnicity, fetalgender and birth weight.

SUPPLEMENTAL FIGURE 3. *The association of maternal FT4 with in the normal range with offspring IQ and gray matter.*



Plots Aand B show the association between maternal FT4 levels during pregnancy within the normal range and child IQ as predicted mean with 95 percent confidence interval (N=3602). Plots C and D show the association between maternal FT4 levels during early pregnancy within the normal range and child MRI brain morphology outcomes as predicted mean with 95 percent confidence interval (N=613).

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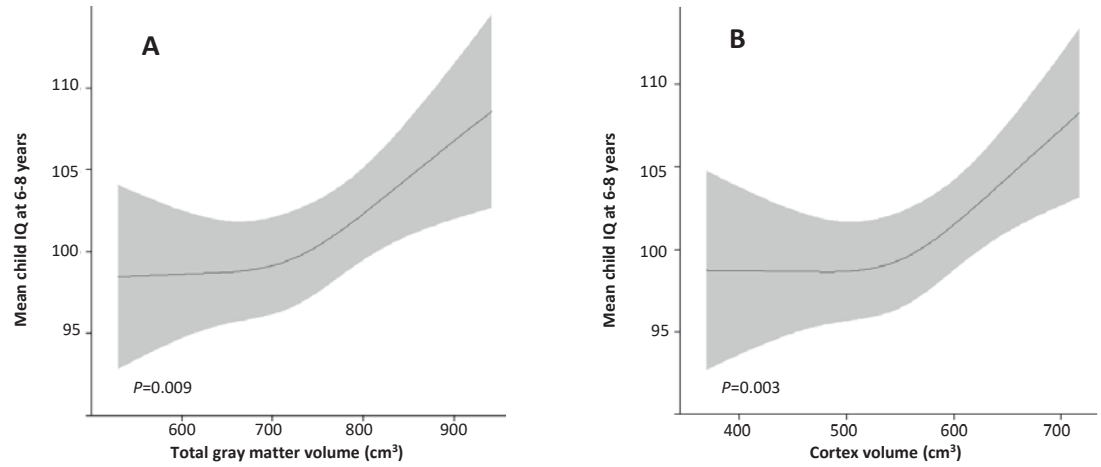
SUPPLEMENTAL FIGURE 4. The association between child total gray volume or cortex volume and IQ.



Plots show the association between child total gray volume (A) or cortex volume (B) and IQ as predicted mean with 95 percent confidence interval. Analyses were performed with R statistical package using the RMS package amongst singleton pregnancies after exclusion of women with IVF treatment (N=76) or women with known thyroid disorders or thyroid interfering medication usage (N=89) and were adjusted maternal age, BMI, parity, education level, child age, gender and birth weight.

SUPPLEMENTAL TABLE 1. *Descriptive statistics of women with gestational FT4 levels >95th percentile.*

		>95th percentile		>5th and <95th percentile	
		Median	**(95% range)**	**Median**	**(95% range)**
TSH	(mU/L)	0.60	(0.00 – 2.81)	1.39	(0.16-4.46)
FT4	(pmol/L)	22.5	(20.8 – 41.8)	14.9	(11.3-19.8)
hCG	(IU/L)	62,052	(16,902 – 142,862)	44,247	(12,384-103,252)
Gestational agea		12.5	(9.1 – 16.8)	13.2	(9.8-17.4)
Maternal age[d]		30.4	(19.8 - 39.5)	30.8	(20.1-39.1)
BMI		23.5	(17.5 - 33.2)	23.5	(18.8-35.5)
Parity[c]					
0		118	(59.3)	2064	(59.7)
1		64	(32.2)	1012	(29.3)
2		14	(7.0)	283	(8.2)
>2		3	(1.5)	98	(2.8)
Smoking[c,e]					
Non-smokers		151	(75.9)	2593	(75.0)
Stopped smokers		22	(11.1)	346	(10.0)
Smokers		26	(13.1)	518	(15.0)
Education level[e]					
None/Primary		292	(7.6)	261	(7.5)
Secondary phase 1		487	(12.7)	443	(12.8)
Secondary phase 2		1203	(31.3)	1072	(31.0)
Higher phase 2		884	(23.0)	798	(23.1)
Higher phase 1		974	(25.3)	883	(25.5)
Ethnicity[c,e]					
Dutch		98	(49.2)	1988	(57.5)
Moroccan		11	(5.5)	178	(5.1)
Turkish		9	(4.5)	242	(7.0)
Surinamese		26	(13.1)	253	(7.3)
Cape Verdian		7	(3.5)	136	(3.9)
Dutch Antilles		4	(2.0)	64	(1.9)
Indonesian		4	(2.0)	107	(3.1)
Asian		5	(2.5)	76	(2.2)
Other western		24	(12.1)	290	(8.4)
Other non-western		11	(5.5)	123	(3.6)
Birth weight (g)		3340	(2331 – 4489)	3455	(2240-4450)
Child gender[c] (boys %)		79	(40.1)	1709	(49.4)

[a] *At time of blood sampling; data shown as median in weeks*
[b] *Data shown as mean in (SD)*
[c] *Data shown as n (%)*
[d] *Data shown as median in years*
[e] *Data shown after imputation of missing data (see methods).*

SUPPLEMENTAL TABLE 2. *Associations of maternal TSH, FT4 with mean child IQ.*

	IQ	
Variables in model	**Beta ± SE[a]**	**P**
TSH	-0.194 ± 0.280	0.50
TSH^2	-0.144 ± 0.059	0.02
FT4	33.81 ± 12.25	0.009
FT4^2	-6.235 ± 2.210	0.007

[a] Reported beta and standard error are increase in cm^3 per log increase in TSH or FT4. TSH^2 and FT4^2 refer to addition of a squared TSH/FT4 variable in the model. Analyses were performed using linear regression models in N=3839 mother-child pairs. TSH and FT4 values were transformed by the natural logarithm. Analyses were adjusted for gestational age at blood sampling, maternal age, smoking, BMI, parity, education level, ethnicity, fetal sex and birth weight.

SUPPLEMENTAL TABLE 3. *Associations between maternal TSH, FT4 and child brain morphology assessed by MRI scanning.*

	Total brain volume		Gray matter volume		Cortex volume		White matter volume		Hippocampal volume	
Variables in model	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE**	**P**
TSH	84.3 ± 27.2	0.002	59.5 ± 17.4	0.0007	54.3 ± 15.3	0.0004	23.8 ± 10.5	0.02	193 ± 213	0.37
TSH^2	-44.7 ± 13.0	0.0006	-31.5 ± 8.32	0.0002	-29.4 ± 7.32	0.00007	-12.7 ± 5.02	0.01	-85.8 ± 102	0.40
FT4	22.6 ± 15.2	0.14	220 ± 97.2	0.02	208 ± 85.6	0.02	12.0 ± 58.6	0.84	236 ± 1185	0.84
FT4^2	-40.2 ± 26.0	0.12	-38.4 ± 16.6	0.02	-36.3 ± 14.6	0.01	-2.78 ± 10.0	0.78	-43.1 ± 202	0.83
	Analyses adjusted for total brain volume									
Variables in model	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE[a]**	**P**	**Beta ± SE**	**P**
TSH	N/A		4.09 ± 4.57	0.37	6.52 ± 4.88	0.18	N/A[b]		-160 ± 187	0.39
TSH^2	N/A		1.78 ± 2.21	0.42	-3.83 ± 2.36	0.11	N/A[b]		104 ± 90.4	0.25
FT4	N/A		81.8 ± 24.7	0.0001	87.6 ± 26.4	0.001	N/A[b]		-688 ± 1019	0.50
FT4^2	N/A		-13.8 ± 4.21	0.001	-14.9 ± 4.51	0.001	N/A[b]		120 ± 174	0.49

[a] Reported beta and standard error are increase in cm^3 per log increase in TSH or FT4.
[b] Not applicable due to high collinearity between total brain volume and white matter volume.
TSH^2 and FT4^2 refer to addition of a squared TSH/FT4 variable in the model.
Analyses were performed using linear regression models in N=646 mother-child pairs. TSH and FT4 values were transformed by the natural logarithm. Analyses were adjusted for gestational age at blood sampling, maternal age, BMI, child sex, birth weight, gestational age at birth and age at time of MRI.

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PART 4: THE INTERPRETATION OF THYROID FUNCTION DURING PREGNANCY

CHAPTER 13

RE-DEFINING TPO-ANTIBODY POSITIVITY DURING PREGNANCY: A FUNCTIONAL, POPULATION-BASED DEFINITION USING AN INDIVIDUAL PARTICIPANT DATA META-ANALYSIS

Tim I.M. Korevaar, Victor J. Pop, Layal Chaker, Mariette Goddijn, Yolanda B. de Rijke, Peter H. Bisschop, Maarten Broere, Vincent W. V. Jaddoe, Marco Medici, Theo J. Visser, Eric A.P. Steegers, Tanja G. Vrijkotte and Robin P. Peeters

Submitted

ABSTRACT

BACKGROUND TPO-antibody (TPOAb) positivity reflects thyroid autoimmunity and is the most important risk factor for (subclinical) hypothyroidism. During pregnancy, the upper limit of TSH to consider levothyroxine treatment differs between TPOAb negative and TPOAb positive women. In other words, the presence of TPOAb positivity has a direct influence on the indication for levothyroxine treatment. Even though all international guidelines recommend population-based pregnancy-specific reference ranges for TSH and FT4, there are no recommendations on the definition of the cut-off for TPOAb positivity. As a consequence, there is a large difference in the definition of TPOAb positivity across populations, assays and hospitals. We aimed to investigate a population-based, pregnancy-specific, functional cut-off to define TPOAb positivity.

METHODS In this individual participant meta-analysis using data from three prospective birth cohorts (Generation R, ABCD study and HAPPY study), we studied the association of TPOAb concentrations with TSH and FT4 concentrations and aimed to identify an effect threshold. Primary analyses were performed on women with available serum samples who presented in early pregnancy (<20 weeks). Data for maternal TSH, FT4 and TPOAbs concentrations, gestational age at birth, and potential confounding factors were obtained.

FINDINGS Data were available for 11 212 women. In all cohorts, TPOAb concentrations were positively associated with TSH concentrations, and negatively associated with FT4 concentrations during early pregnancy (all $P<0.0001$). TSH concentrations were higher at TPOAb concentrations from the 92nd percentile upwards. The risk for a TSH concentration above 2.5 mU/L was also higher at TPOAb concentrations from the 92nd percentile onwards with absolute risks ranging between 19.4% to 51.3% compared to 8.3% in the reference group. The thyroidal response to hCG stimulation was also lower from the 92nd percentile upwards.

Subsequent analyses indicated that there was effect modification by TPOAb cut-offs of the association of TSH concentrations with premature delivery. Stratified analyses showed that women with TPOAb concentrations below manufacturer cut-offs already had a higher risk of premature delivery, especially when TSH concentrations were high(-normal).

In the total study population, 22.7% of women with TPOAb concentrations associated with higher TSH were not considered TPOAb positive according to manufacturer-based cut-offs. Of these women, 213 had a TSH between 2.5 and 4.0 mU/L, but 40 (18.8%) women would not be eligible to receive levothyroxine treatment based on the new ATA guidelines as they would be considered TPOAb negative according to manufacturer cut-offs.

INTERPRETATION We show that a considerable proportion of women with TPOAb concentrations high enough to affect thyroid function are currently considered TPOAb negative. This study suggests that the currently used cut-offs for TPOAb positivity may be too high. Furthermore, the use of a population-based cut-off for TPOAbs may identify women with a clinically relevant extent of thyroid autoimmunity and have a higher risk or premature delivery, but that would otherwise not be considered TPOAb positive or eligible for treatment.

INTRODUCTION

Maternal thyroid hypofunction during early pregnancy is associated with miscarriage, premature delivery and suboptimal offspring neurobehavioral development and disease.[1-5] Thyroid autoimmunity, reflected by thyroperoxidase antibody (TPOAb) positivity, is the most important risk factor for low thyroid function and reported to occur in about 5.6-22.1% of all pregnant women worldwide.[6-15] Thyroid autoimmunity causes a gradual decrease of the functional capacity of the thyroid which leads to an increase in TSH and decrease in FT4 concentrations, and ultimately causes hypothyroidism. However, well before the onset of changes in thyroid function, a subclinical decrease in thyroid functional capacity may be present. This decreased capacity may become apparent during a state of increased demand such as early pregnancy, when high concentrations of human chorionic gonadotropin (hCG) stimulate the thyroid. As such, the adverse effects of thyroid autoimmunity on thyroid function may be amplified during early pregnancy.

Recent studies indicate that TPOAb positive women have a higher risk of adverse outcomes, particularly when TSH concentration are (mildly) elevated [3, 6, 8, 16] and that this may be overcome with levothyroxine treatment.[17, 18] For this reason, the guidelines of the American Thyroid Association (2016) recommend consideration of levothyroxine treatment in TPOAb positive women with a TSH concentration above 2.5 mU/L, as opposed to a TSH concentration above a population-based reference range for TPOAb negative women.

Although all international guidelines advocate the use of pregnancy-specific references ranges for TSH and FT4 measurements during pregnancy, there are no recommendations for the definition of TPOAb positivity.[19-21] Instead, cut-offs provided by the assay manufacturers are usually used. However, such cut-offs are derived utilizing non-pregnant study subjects and/or according to different definitions (for example population reference ranges or the sensitivity of the assay). As a consequence, a wide range of cut-off values (ranging from 15 to 143 IU/L) are used to define TPOAb positivity. This has resulted in divergent estimates of the prevalence of TPOAb positivity during pregnancy and non-generalizable reported risk estimates for adverse pregnancy outcomes in TPOAb positive women.[6-15] There are practically no data available about the threshold from which TPOAb concentrations affect thyroid function during pregnancy while the cut-offs used to define TPOAb positivity during pregnancy may directly determine whether a patient is eligible for levothyroxine treatment or not.

The main aim of this study was to identify an optimal, pregnancy-specific, cut-off value for TPOAb positivity based on changes in thyroid function using a population-based approach across three large prospective Dutch birth cohorts.

MATERIALS AND METHODS

Design

Women were included from the Generation R Study, a population-based prospective birth cohort from early fetal life onwards in Rotterdam[22], the Amsterdam Born Children and their Development (ABCD) study, a population-based prospective birth cohort from Amsterdam[23], and the Holistic Approach to Pregnancy and the first Postpartum Year (HAPPY) study, a population-based prospective birth cohort from the Eindhoven area.[24]

In Generation R, a total of 7069 women living in the Rotterdam area with a delivery date between April 2002 until January 2006 were enrolled during early pregnancy (<18 weeks) in hospitals and midwife practices.[22] Blood samples were drawn at first presentation in 6398 of these women and TPOAbs and

TSH or FT4 concentrations were measured in 5563 women (missing due to lack of adequate serum volume). Women with twin pregnancies (N=128), women that underwent fertility treatment (N=76), women with pre-existing thyroid disease or thyroid (interfering) medication usage (N=89) were excluded. The iodine status of the Netherlands is generally iodine sufficient, as reflected by the iodine status in the Generation R study (median population urinary iodine 225 µg/L).[25]

In the ABCD study, all pregnant women living in Amsterdam and had their first visit to an obstetric caregiver between January 2003 and March 2004 were eligible to be included in the study and invited to participate. In total, 12377 pregnant women were asked to participate and 8266 women agreed (response rate 67%). Additional informed consent for blood collection was given by 4389 women (53%). TSH, FT4 and TPOAb concentrations were measured for 4079 women (<20 weeks). Women with twin pregnancies (N=50), pre-existing thyroid disease or thyroid (interfering) medication usage (N=32) or fertility treatment (N=110) were excluded.

In the HAPPY study, eligible mothers were those who presented at any of 17 primary care community midwife practices in the area of South-East Brabant (the Netherlands), from January 2013 until September 2014.[24] A total of 2103 women with a singleton pregnancy were enrolled and in all women blood samples from early pregnancy (<20 weeks) were available. In 1712 women, a blood sample from late pregnancy was also available. TSH, FT4 and hCG were measured during early pregnancy and during late pregnancy (median week 32, 95% range: 31-35) in all women. Women with pre-existing thyroid disease or thyroid (interfering) medication usage (N=47) were excluded, no data was available on fertility treatment. Further details on data ascertainment are presented in the supplemental appendix.

Exposures, outcomes and potential confounders

Details of TSH, FT4, TPOAb and hCG measurements are described in the supplemental appendix. In short, three different assays were used to measure TPOAbs (Generation R: Phadia 250; ABCD: ELIZEN TG Ab (E-CK-96); and HAPPY: Roche Cobas e601), three different assays were used to measure thyroid function, (Generation R: Vitros ECI (Ortho Clinical Diagnostics); ABCD: Acces (Beckman Coultier); and HAPPY: Roche Cobas e601), hCG was available in two cohorts (Generation R and HAPPY) and measured with two different assays(Generation R: Immulite 2000 (Siemens) and HAPPY (Roche Cobas e601)). In addition, serum measurements were performed in Generation R and ABCD while plasma measurements were performed in HAPPY. These differences reflect the differences between laboratories and clinical practices worldwide. In order to cope with thesedifferences, we used population-based percentiles for TPOAbs, similar to what is recommended for thyroid function measurements during pregnancy by international guidelines.[19-21] Even though the absolute value for the TPOAb concentrations may differ largely between different measurement methodologies, there is a high inter-methodology correlation, particularly for population-based percentiles. Details on data ascertainment of other covariates are provided in the supplemental appendix.

In women with the same hCG concentration, a lower FT4 concentrations may be a sign of a lower thyroidal response to hCG stimulation. We defined the thyroidal response to hCG stimulation cross-sectionally based on the assumption that the predicted means in the whole population are the best approximation of hCG mediated FT4 changes in an individual (i.e. the expected thyroidal response). The thyroidal response to hCG stimulation was defined by the residuals of a regression model in which hCG was regressed on FT4 or TSH. The residuals of these associations reflect the expected thyroidal response to hCG, i.e. the distance (in SD) of deviation from the mean (i.e. expected value) of the association of hCG with TSH or FT4 (see Supplemental Figure 1 for a graphical depiction of this concept).

Data on gestational age at birth was obtained from community midwives, obstetricians, and hospital registries and was available for 11,053 out of 11,212 of the included individuals (98.6%). There was no

difference in TSH or TPOAbs between the groups with and without data available for gestational age at birth. Premature delivery was defined as the onset of premature labor before the 37th gestational week.

Statistical analysis

To fulfill model assumptions and to better reflect biological differences, TSH and TPOAb concentrations were logarithmically transformed. Sensitivity analyses were performed to detect outliers in each cohort by excluding subjects with TSH or FT4 concentrations higher than +3SD or below -3SD (after log transformation for TSH). Non-linearity was assessed using ordinary least squares linear regression methods utilizing restricted cubic splines with 3 knots at the 10th, 50th and 90th percentile. For all analyses, model fit and remaining model assumptions were assessed by plotting model residuals, evaluating (adjusted) R-squared and/or the le Cessie - van Houwelingen - Copas - Hosmer unweighted sum of squares test. We considered gestational age at blood sampling, maternal age, BMI, ethnicity, parity, smoking status, education level and fetal sex as potential confounders and these covariates were added to the models based on biological plausibility, change in the effect estimates of interest and reduction in the residual variability of the outcome.

First, we studied the association of TPOAb concentrations with TSH and FT4 concentrations using multiple linear regression models for each cohort separately. Second, we investigated this association using population-based percentiles for TPOAb concentrations and population-based SD values for TSH and FT4 by performing an individual participant meta-analysis of the three cohorts. We screened for a threshold of this association using an unrestricted multiple linear regression model (utilizing 10 restricted cubic splines), which indicated an effect threshold at least above the 80th percentile. Subsequently, to more specifically define a threshold for the effects of TPOAbs on TSH or FT4, we studied the mean TSH and FT4 concentration for each percentile of TPOAbs starting at the 80th percentile. We subsequently utilized multiple logistic regression models, using a similar approach, to study threshold effects of the association of TPOAbs with the risk of TSH > 2.5 mU/L. The latter analysis was performed in women with a TSH<4.0 mU/L because women with TSH concentrations above this threshold are already eligible to receive treatment according to the new ATA guidelines.

We compared multiple linear or logistic regression models (with or without adjustment for cohort as a categorical variable) with multilevel models (with random slopes and/or intercepts) for studying the association of TPOAbs with TSH, FT4 and TSH > 2.5mU/L. Standard linear regression models (without adjustment for cohort, but still using cohort-standardized variables) were superior to the use of multilevel models based on Akaike information criterions and log-likelihood tests. Furthermore, there was no interaction of cohort with TPOAbs in any of the models and similar results were obtained when each cohort was assessed separately. We studied if the association of TPOAb concentrations with TSH and FT4 concentrations were different according to the week of gestation at blood sampling by adding a product interaction term to the model (gestational age at blood sampling*percentile TPOAb) in the full study population, in a study population of women with TPOAbs > 80th or >90th percentile while taking into account potential non-linear effects. In addition, we also stratified analyses according to the median (13 week gestation) to optimize statistical power. We studied if the association of TPOAb percentiles with premature delivery differed by TSH concentrations by adding a product interaction term to the model (log(TPOAbs+1)*TSH and TPOAb percentiles*TSH). Given the sparse occurrence of premature delivery, high TSH and high TPOAbs, we considered a *P*-value for interaction of <0.15 for subsequent stratified analyses to quantify clinical relevance.[26] Analyses were arbitrarily stratified per population percentile for TPOAbs and according to different cut-offs of high(-normal). We used women within the interquartile range for TSH and below the 80th percentile of the population TPOAb percentile as the reference group. There was a non-specific peak in premature delivery between percentile 91

and 92 (OR 1.99, *P*=0.03) and to overcome this we additionally stratified per half percentile. Multiple imputation according to the Markov Chain Monte Carlo method was used for missing data on covariates, a model with relevant covariates was constructed for each cohort, five imputed data sets were created and pooled for analyses.[27] Further details are described in the Supplemental Appendix. All statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA) or using R statistical software version 3.03 (packages *rms*, *hmisc, mice* and *visreg*).

RESULTS

After exclusions, the final study population comprised 11 212 women included during early pregnancy (median gestational age at blood sampling: 13.0 weeks [95% range: 9.1-17.6]) and 1 665 women during late pregnancy (HAPPY study, median gestational age at blood sampling: 32.3 weeks [95% range: 31.1-35.5]) as is shown in Figure 1. Women were predominantly of Dutch origin, primiparous and non-smokers, further descriptive characteristics are shown in Supplemental Table 1.

There was a positive association of TPOAb concentrations with TSH concentrations during early pregnancy in all cohorts (all *P*<0.0001; Figure 2A-C). There was a negative association of TPOAb concentrations with FT4 concentrations during early pregnancy in all cohorts (all *P*<0.0001; Figure 2E-G). During late pregnancy, there was a positive association of TPOAb concentrations with TSH concentrations (*P*<0.0001; Figure 2D). However, compared to early pregnancy, the association of TPOAb concentrations with TSH concentrations was attenuated by approximately 60% (Figure 2C-D). There was no association of TPOAb concentrations with FT4 concentrations during late pregnancy (*P*=0.39; Figure 2H).

Assessment of a population-based percentile cut-off for TPOAb concentrations

Subsequently, we investigated from which percentile of TPOAb concentrations, TSH and FT4 concentrations start to shift. Higher mean TSH concentrations were observed from TPOAb concentrations of the 92nd percentile upwards and lower mean FT4 concentrations were observed from the 94th percentile upwards during early pregnancy (Figure 3). Similar percentile cut-offs were found when each cohort was analyzed separately (Supplemental Figure 2A-B). Compared to currently used cut-offs for TPOAb positivity, a population-based cut-off of >92nd differed considerably in Generation R (manufacturer 60 IU/L (94.4th percentile) versus population-based 25.7 IU/L) and in ABCD (manufacturer 80 IU/L (94th percentile) versus population-based 30.7 IU/L), but not in HAPPY (manufacturer 35 IU/L versus population-based 34 IU/L; Table 1).TSH and FT4 concentrations did not differ per percentile cut-off for TPOAb concentrations during late pregnancy (Supplemental Figure 3).

FIGURE 1. *The risk of premature delivery according to TPOAb cut-offs and TSH concentrations.*

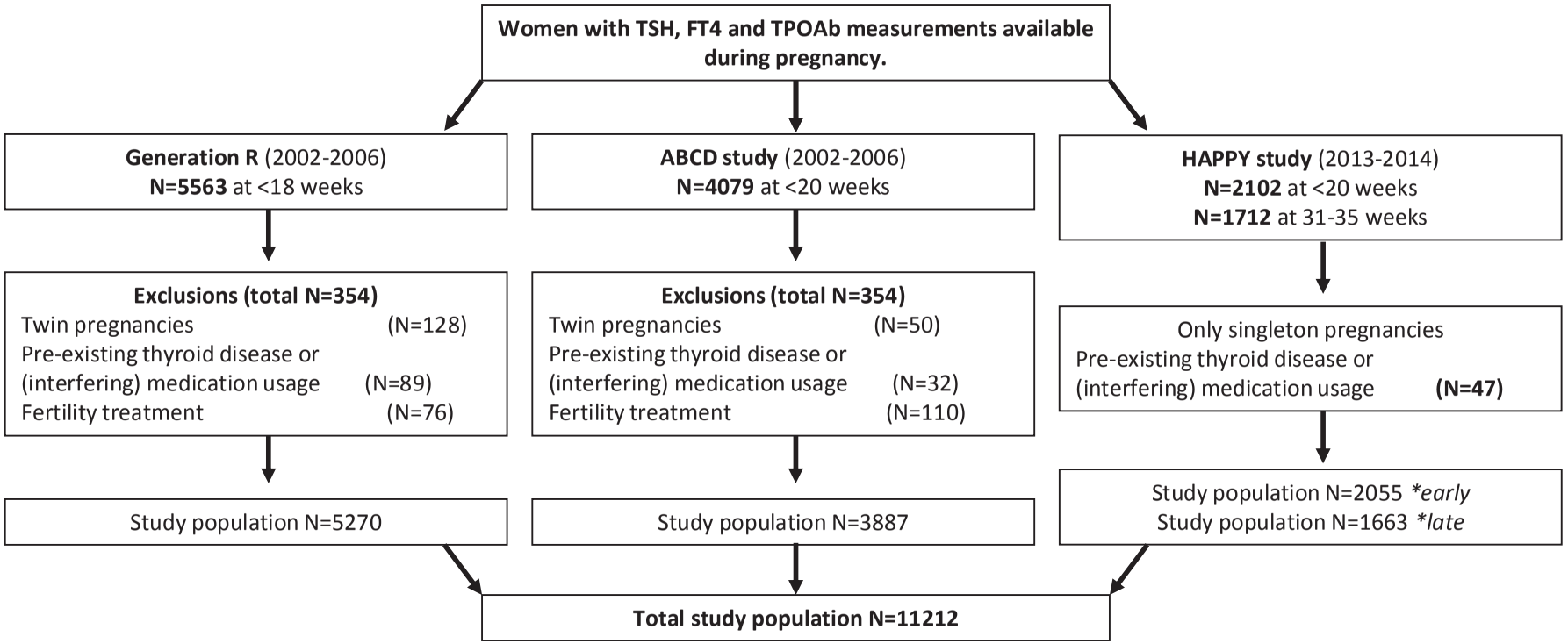


FIGURE 2. Association of TPOAb concentrations with TSH and FT4 during early and late pregnancy.

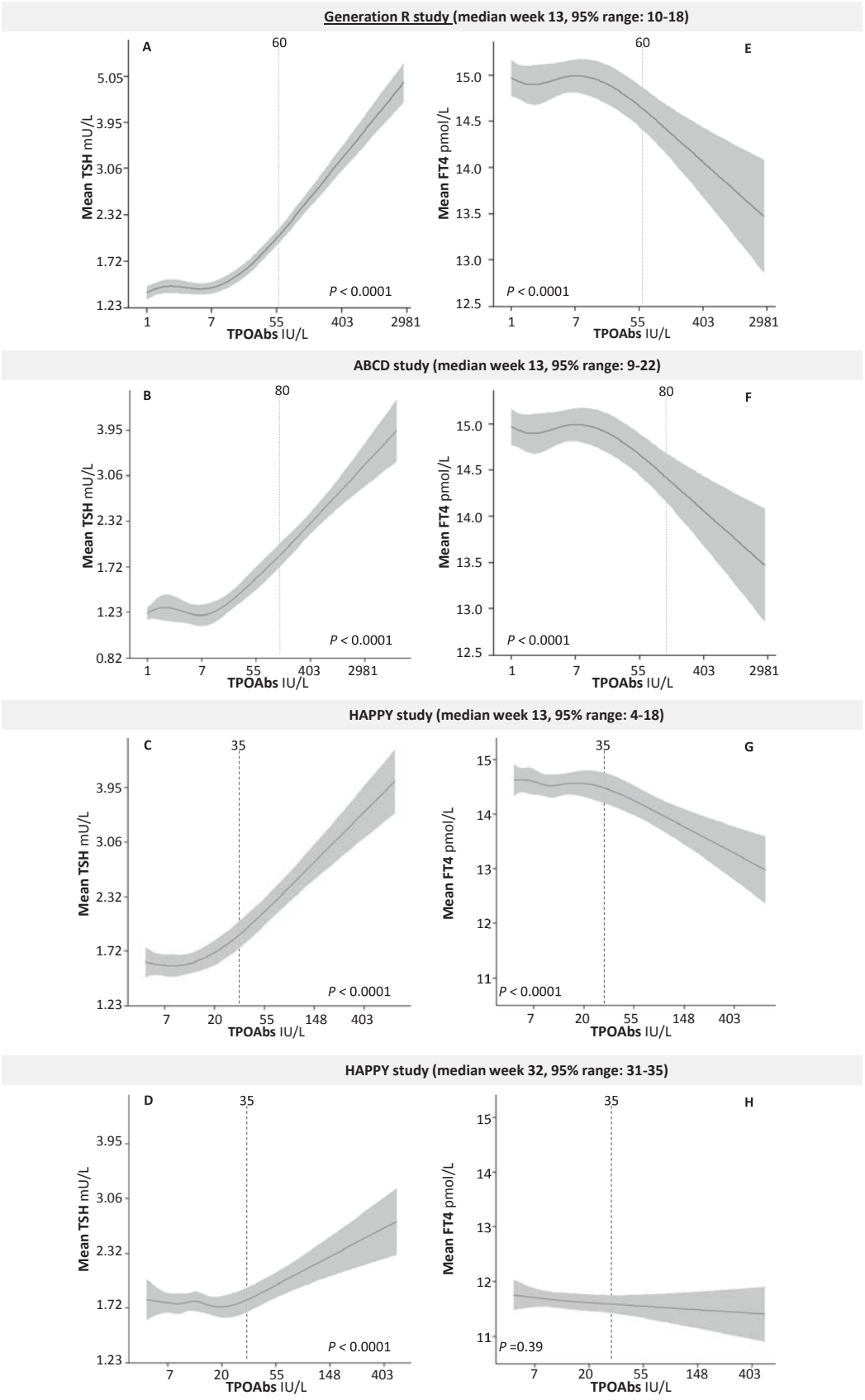


Figure 2. shows the association of TPOAb concentrations with mean TSH (A-D) and FT4 (E-H) during early pregnancy as estimated mean (black line) with 95% confidence interval (grey area) per cohort. The vertical lines show cut-offs for TPOAb positivity as provided by the manufacturers (35, 60 or 80 IU/L). All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

TABLE 1. *Difference in TPOAb positivity and treatment eligibility between manufacturer an population-based reference ranges for TPOAb positivity.*

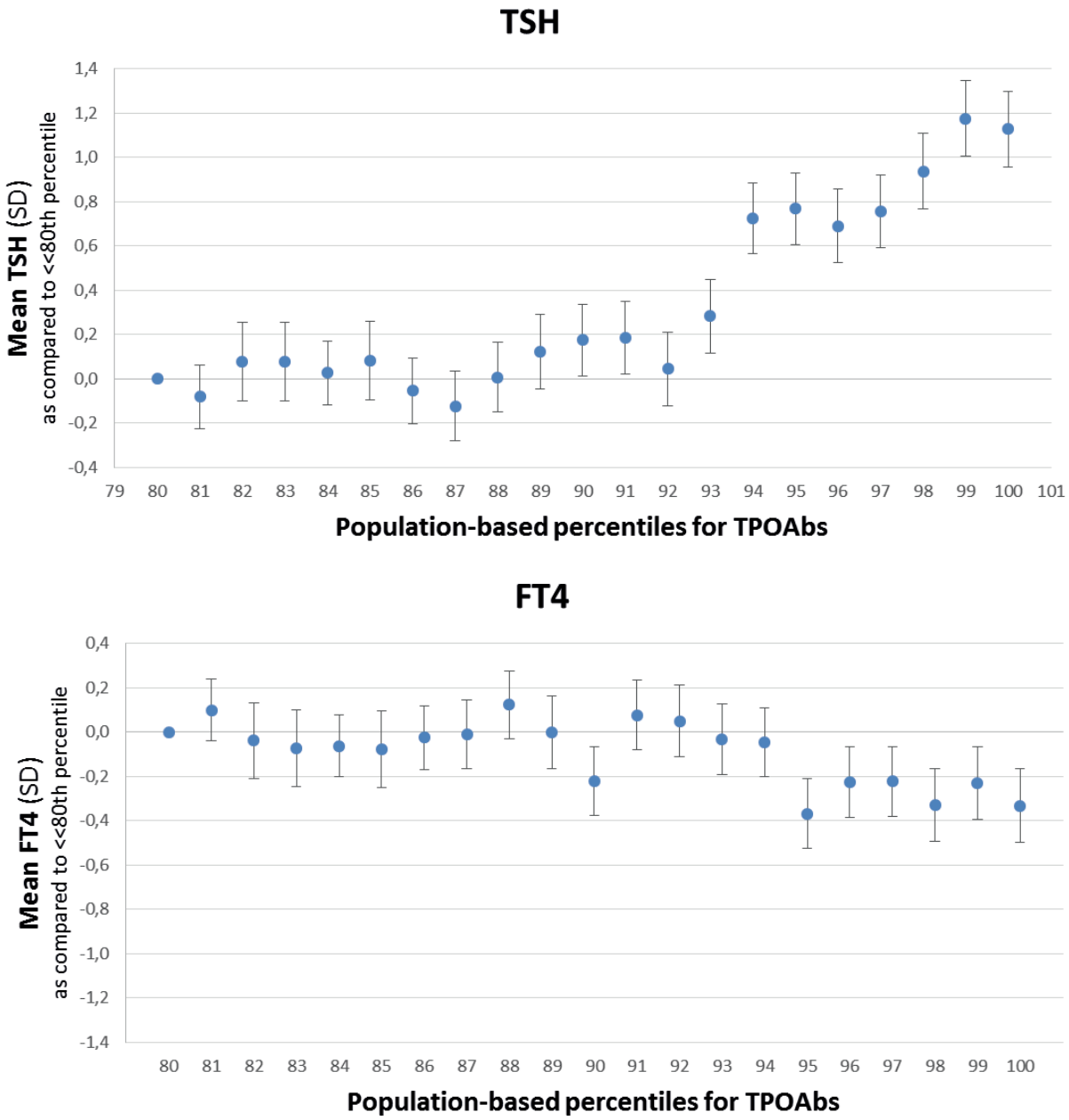
		TPOAb positivity diagnosis		Treatment eligibility[a]	
				Women with TSH <4.0 mU/L	
TPOAb cut-off	**(IU/L)**	**TPOAb positive**	**Difference**	**Eligible**	**Not eligible**
Generation R*					
Manufacturer	**(60)**	296 (5.7)	**123 (29.4)**	85 (1.7)	**26 (23.4)**
≥ 92nd percentile	**(25.7)**	419 (8.1)		111 (2.2)	
ABCD					
Manufacturer	**(80)**	235 (6.0)	**82 (25.9)**	43 (1.1)	**14 (24.6)**
92nd percentile	**(30.7)**	317 (8.2)		57 (1.4)	
HAPPY					
Manufacturer	**(35)**	162 (7.9)	**2 (1.2)**	45 (2.3)	**0**
≥ 92nd percentile	**(34)**	164 (8.0)		45 (2.3)	
Total					
Manufacturer		693 (6.2)	**207 (22.7)**	173 (1.6)	**40 (18.8)**
≥ 92nd percentile		910 (8.1)		213 (1.9)	

* To allow comparison, only women with TPOAbs and TSH measurements available were selected (N=5195/5270)
[a] Based on most recent guidelines (ATA guidelines 2016)
Manufacturer as compared to population-based cut-offs.

The role of pregnancy-specific physiology

During early pregnancy, high hCG concentrations effectuate an increase in FT4 and a subsequent decrease in TSH. We have recently shown that women with thyroid autoimmunity (TPOAb positivity defined according to manufacturer-based cut-offs in Generation R and HAPPY) have an attenuated thyroidal response to hCG stimulation. Therefore, we investigated from what population-based TPOAb cut-off the thyroidal response to hCG would start to attenuate (see methods section and Supplemental Figure 1 for further details). Higher TPOAb concentrations were associated with an impaired TSH and FT4 response to hCG stimulation, in both cohorts (hCG data available in Generation R and HAPPY, both *P*<0.001; Supplemental Figure 3). Similar to the analyses on mean TSH and FT4 concentrations, these effects became apparent from the 92nd percentile upwards for TSH and from the 94th percentile upwards for FT4 (Supplemental Figure 4).

FIGURE 3. *Population-based TPOAb percentiles and mean TSH and FT4 during early pregnancy.*



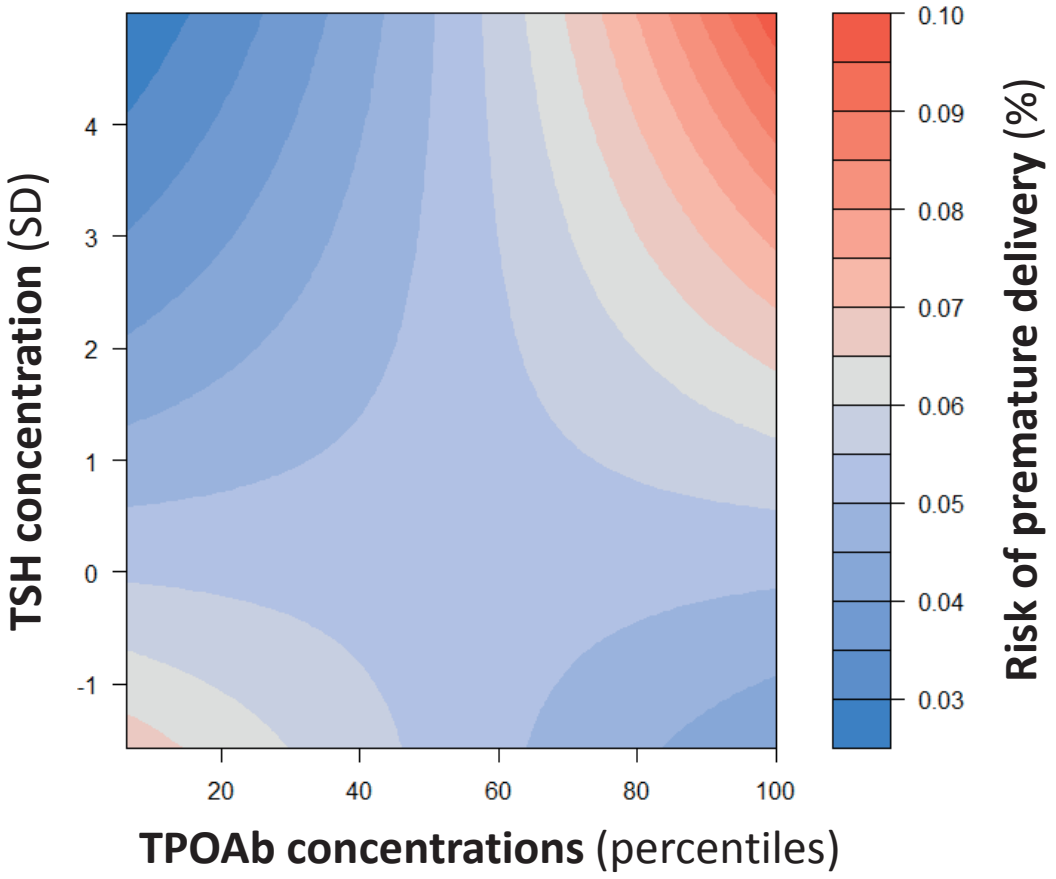
Plots show the mean (±95% CI) difference in TSH and FT4 for each population-based percentile of TPOAbs as compared to the reference group (≤P 80). Group size ranged between 108 and 114. All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

The risk of premature delivery

TPOAb positivity (based on manufacturer cut-offs) has been consistently associated with a higher risk of premature delivery. Furthermore, recent studies have suggested that the combination of TPOAb positivity with high-normal TSH concentrations is associated with a synergistically higher risk of adverse outcomes (including premature delivery) most likely because higher TSH concentrations are a reflection of more severe thyroid autoimmunity. Therefore, we subsequently analyzed the risk of premature delivery according to TPOAb positivity percentiles and TSH concentrations. The association of TPOAb

percentiles with premature delivery was modified by TSH concentrations (*P* for interaction TSH*TPO percentiles=0.064 and TSH*log(TPO)=0.050; Figure 4). Subsequently, stratified analyses showed that TPOAb concentrations below the manufacturer cut-offs were associated with a higher risk of premature delivery, especially when TSH concentrations were high (Figure 4; and Supplemental Table 2).

FIGURE 4. *The risk of premature delivery according to TPOAb cut-offs and TSH concentrations.*



	Odds ratio for premature delivery							
TSH > p95	1.27	1.27	3.36	4.60	2.17	2.20	2.26	2.17
TSH > p90	1.16	2.57	3.66	6.46	1.88	1.91	1.94	1.87
TSH > p85	1.01	1.89	3.14	7.03	1.61	1.63	1.67	1.61
TSH > p80	1.05	2.01	3.20	4.95	1.49	1.51	1.54	1.48
TSH > p75	1.02	1.77	2.53	3.72	1.48	1.50	1.54	1.48
Cut-offs	**TSH overall**	**p85**	**p90**	**p91**	**p92**	**p93**	**p94**	**Manufacturer**

Figure 4 Displays a heat map for the risk or premature delivery (red color indicates high risk. blue color indicates low risk) according to the interaction between TPOAb cut-offs (cohort-specific percentiles for TPOAbs) and TSH concentrations (cohort-specific SD scores for TSH scores). Interaction was tested after excluding outliers (ie.g. women with overt hypo. overt hyperthyroidism or TSH >10 mU/L). Manufacturer cut-offs were 60 IU/L for Generation R. 80 IU/L for ABCD and 35 IU/L for HAPPY. TSH cut-offs corresponded to the following TSH concentrations for respectively Generation R. ABCD and HAPPY: P95 (3.67. 2.83. 3.73). P90 (2.90. 2.31. 2.94). P85 (2.51. 2.03. 2.59). P80 (2.24. 1.85. 2.28). P75 (2.03. 1.69. 2.08).

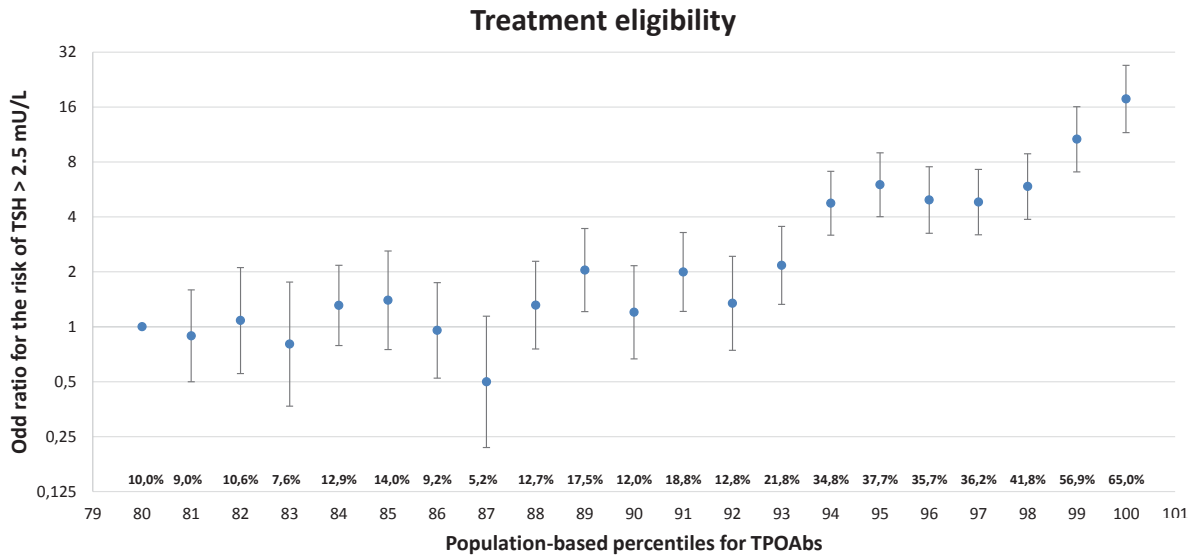
Assessment of treatment eligibility according to ATA guidelines

In TPOAb positive women, according to the new ATA guidelines, treatment can be considered at TSH concentrations above 2.5 mU/L while a TSH concentration above a population-based reference range or

roughly 4.0 mU/L is recommended for TPOAb negative women. The proportion of women with a TSH of 2.5-4.0 mU/L per cohort was: 578/4977 (11.6%) in Generation R, 228/3792 (6.0%) in ABCD and 249/1981 (12.6%) in HAPPY. In women with TSH concentrations below 4.0 mU/L, the risk of a TSH concentration above 2.5 mU/L became higher from the 92nd percentile upwards. Risk estimates indicated a 2.3 to 19.2-fold increased risk (Figure 5; $P \leq 0.0017$). The corresponding absolute percentage of women with a treatment indication changed from 8.3% (reference) to 19.4% to 51.3% above this cut-off (Figure 5).

In the total study population, 910 (8.1%) of all women had a TPOAb concentrations that was associated with higher TSH concentrations, of which 207 (22.7%) were not considered TPOAb positive according to manufacturer-based cut-offs. (Table 1). Furthermore, 213 of the 910 women (23.4%) with TPOAb concentrations that was associated with a higher TSH concentration had a TSH between 2.5 and 4.0 mU/L. Of these 213 women, 40 (18.8%) women were not considered TPOAb positive and would not be eligible to receive levothyroxine treatment according to the new ATA guidelines (Table 1).

FIGURE 5. *Population-based TPOAb percentiles and treatment eligibility.*



Plots show the odds ratio (±95% CI) for the risk of TSH > 2.5 mU/L for each population-based percentile of TPOAbs. as compared to the reference group (≤P 80). Group size ranged between 108 and 114. All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity. In addition, the absolute risks in the combined study population are given as percentages.

DISCUSSION

In this large, individual participant-based meta-analysis of three prospective Dutch birth cohorts we investigated the optimal cut-off to define TPOAb positivity during pregnancy based on changes in thyroid function. To our knowledge, this is the first study to show that current cut-offs for TPOAb positivity may fail to identify up to 29.4% of women with TPOAb concentrations high enough to affect thyroid function. Consequently, for a considerable proportion of these women (those with a TSH between 2.5 and 4.0 mU/L) the indication for levothyroxine treatment changes. We demonstrate by using a population-based and functional approach to define a cut-off for TPOAb positivity that a higher mean TSH concentration, a higher risk of TSH >2.5 mU/L and a change in the thyroidal response to hCG

stimulation all occur from the 92nd percentile onwards. We also demonstrate that women with TPOAbs below currently used cut-offs already have a higher risk of premature delivery and that this risk differs based on the TSH concentration.

TPOAb positivity is the most important risk factor for low maternal thyroid function during pregnancy. TPOAb positivity is associated with higher TSH concentrations and the combination of TPOAb positivity with mildly elevated TSH concentrations (i.e. from 2.5 mU/L upwards) is associated with a synergistically higher risk of adverse outcomes.[1, 3, 6, 8] In addition, randomized controlled trials indicate that levothyroxine treatment in TPOAb positive women may be beneficial, and that this benefit is predominantly evident in women with higher TSH concentrations.[17, 18] In the current study, we demonstrate that TPOAb concentrations are associated with a higher TSH concentration as well as a higher risk of a TSH concentration above 2.5 mU/L from a population-based cut-off the 92nd percentile upwards. These findings were highly similar across all three populations indicating that a pregnancy-specific, population-based derived cut-off can optimize the definition of TPOAb positivity and thus the identification of clinically relevant thyroid autoimmunity. In addition, we also show that women below currently used cut-offs for TPOAb positivity have a higher risk of premature delivery. These findings can help to improve the identification of clinically relevant thyroid autoimmunity in pregnant women and are in line with recommendations from international guidelines on reference ranges for thyroid function during pregnancy.[19-21] Larger studies are needed to define the optimal cut-offs for the combination of TPOAb cut-offs and TSH concentrations based on the risk of adverse outcomes.[28]

Currently used strategies to define the cut-off for TPOAb positivity include population-based reference range calculations in healthy non-pregnant individuals as well as the sensitivity of the assay to detect TPOAbs. These strategies are complicated by a considerable inter-method variability of TPOAb assays (range correlation coefficients: 0.65-0.87) and differences in the purity of the TPOAb reagent.[29] In the current study, we show that cut-offs currently used for various TPOAb assays may fail to diagnose TPOAb positivity during pregnancy in up to 29.4% of cases and, according to the new ATA guidelines, this affects the indication for levothyroxine treatment in 18.8% of women. Our data demonstrate that manufacturer based cut-offs are adequate during early pregnancy for the HAPPY study (34 IU/L vs. 35 IU/L; assay: Cobas e601, Roche), but are likely to be too high for Generation R (60 IU/L vs 25.7 IU/L; assay: Phadia 25, Phadia) and the ABCD study (80 IU/L vs 30.7 IU/L; assay: E-CK-96, ELIZEN). In addition, we also demonstrate that large between-cohort differences exist in the proportion of women with an absolute TSH concentration between 2.5 and 4.0 mU/L, most likely due to assay differences (from 6.0% in ABCD, to 11.6% in Generation R and 12/6% in HAPPY). Because these numbers were highly variable between the cohorts, further studies are required to quantify the extent of under diagnosis for other TPOAb assays.

From a public health perspective, the proportion of young women that we consider to be TPOAb positive during early pregnancy of 8.1% is relatively large. This is most likely caused by pregnancy-specific changes in thyroid physiology that affect the functionally defined cut-off more than in a non-pregnancy state. hCG exerts thyrotropic activity and high hCG concentrations during early pregnancy stimulate the thyroid gland leading to an increase in FT4 concentrations and a subsequent decrease in TSH concentrations.[30] As such, thyroid autoimmunity may become apparent upon thyroidal stimulation during pregnancy in women with an otherwise normal thyroid function. A recent study from our group showed that the thyroidal response to hCG stimulation is considerably attenuated in TPOAb positive women (defined by manufacturer cut-offs).[31] In the current study we show the thyroidal response to hCG stimulation actually starts to attenuate from a lower TPOAb concentration (<92nd percentile) than the manufacturer cut-offs. This new threshold is similar to the threshold from which a shift in TSH concentrations starts to occur. Furthermore, we also demonstrate that TPOAbs are not associated with

changes in TSH or FT4 concentrations during late pregnancy when hCG concentrations are substantially lower.

We found that a shift in mean TSH concentrations occurred from a different TPOAb concentration than changes in FT4 (92nd and 94th percentile, respectively). Most likely, we were able to detect effects on TSH concentrations more easily because of the because of the log-linear relationship between TSH and FT4, because of which small changes in FT4 concentrations effectuate larger relative changes in TSH concentrations.[32] This is also supported by our results showing that higher TPOAb concentrations are associated with a much larger relative change in TSH concentrations (~200% to ~300%) than FT4 concentrations (~10%). In addition, the initial increase in TSH concentrations may attenuate the association of TPOAb concentrations with FT4 concentrations. These findings suggest that TSH concentrations are the preferred marker for the assessment of thyroid function changes due to thyroidal autoimmunity and the addition of TSH may add to the specificity of a TPOAb positivity cut-off.

Previous studies that investigated a cut-off value for TPOAb concentrations did not use a population-based approach, did not take into account thyroid function, lacked external replication and/or were not generalizable to a pregnant population. Our study investigated the association of TPOAb concentrations with TSH and FT4 during early and late pregnancy and identified a functional, population-based threshold for TPOAb positivity. In contrast to the replicated population-based cut-offs reported on in this paper, the generalizability of the proportions of women that are under diagnosed or lack treatment despite a treatment indication is limited to the assay combinations used in the current study. Further studies in larger cohorts are required to provide data for other assays as well.[28]

Although we had a large study population, the interpretation of risk estimates for premature delivery stratified per TPOAb percentile and high TSH cut-off should be interpreted with care as each stratum only includes a small number of women that had a premature delivery. However, we were able to replicate previous findings that show that women with the combination of high TPOAb concentrations with high-normal TSH concentrations have a synergistically higher risk of premature delivery[3, 6, 8, 16] using a continuous analysis. Given the low prevalence of TPOAb positivity, high TSH concentrations and premature delivery (roughly 8%, 5-10% and 5-10%, respectively), larger studies are needed to adequately define TPOAb and TSH thresholds for disease risk.

Furthermore, it is important to note that the generalizability of our results is limited to pregnancy week 8-20 and 31-35 since women outside of these weeks were underrepresented or absent in this study. However, it is important to note that in clinical practice, the first clinical assessment in the majority of pregnant women will take place during the timeframe of early pregnancy in which this study was conducted. Our results suggest that thyroid autoimmunity impairs the thyroidal stimulation by hCG and therefore that early pregnancy specific cut-offs for TPOAbs are required. In addition, both low and high iodine intake are associated with thyroid autoimmunity.[33] Since the Netherlands is an iodine sufficient country, further data are required to study optimal TPOAb cut-offs in areas with mild-to-moderate iodine deficiency.

In conclusion, this analysis of a population-based cut-off for TPOAb positivity shows that from the 92nd percentile upwards, TPOAb concentrations are associated with an adverse shift in thyroid function during early pregnancy. Based on currently used manufacturer-based cut-offs, a large proportion of women with TPOAb concentrations that adversely affect thyroid function are currently considered to be TPOAb negative, which has consequences for clinical risk stratification and levothyroxine treatment indication. These data suggest that a change in the definition of TPOAb positivity can improve the identification women at high risk and potentially contribute to the prevention of adverse pregnancy outcomes related to thyroid autoimmunity. Further studies are needed to replicate these findings for other TPOAb, TSH and FT4 assays and to further define optimal thyroid status during pregnancy.

SUPPLEMENTAL APPENDIX - METHODS

Generation R Study

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (median 13.2 weeks; range 4.5-17.9). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L. TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/ml.

Determinants and covariates

Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[1] Information on maternal age, smoking status, maternal education level, obstetrical history and ethnicity was obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands and grouped according to major ethnic groups in the Netherlands and/or similarity in thyroid function tests.[2 3] Maternal smoking status was classified as no smoking, smoking until known pregnancy (stopped), and continued smoking during pregnancy. Education level was defined according to finished education as none/primary, secondary or higher. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). Information on fertility treatment and gender of the child were obtained from community midwives, obstetricians, and hospital registries. Medical and obstetrical history were assessed by questionnaires and answers were crosschecked by certified medical doctors. As we determined previously, this study population is iodine sufficient.[4]

Multiple imputation

In Generation R, maternal smoking, education level, ethnicity, hCG, parity and BMI were imputed (missing due to non-response in 13.1%, 7.2%, 3.2%, 4.1%, and <1.0%, respectively).

ABCD study

Thyroid measurements

Maternal serum samples were obtained during early pregnancy (12.7 weeks; range 4.1-18). Plain tubes were centrifuged and serum was stored at -80 C. TSH and FT4 were determined in maternal serum samples using an access immunoanalyser (Beckman Coultier, Inc.). The intra- and interassay coefficients of variation were <5.0% for TSH and 3.1-5.0% for FT4. TPOAbs were measured using an enzyme-linked immunosorbent assay (ELISA) [ELIZEN TG Ab (E-CK-96), Zentech, Luik, Belgium] and considered positive when >80 IU/ml.

Determinants and covariates

Data on gestational age at blood sampling, parity, child gender, obstetrical history and twin pregnancies were derived from the Youth Healthcare Registration. Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating (~90%) or according to self-reported last menstrual period. Information on maternal age, weight, length

(used to calculate BMI), smoking status, years of maternal education, ethnicity, pre-existing thyroid disease, fertility treatment and thyroid interfering medication usage were obtained by questionnaires during pregnancy. Ethnicity was determined by country of origin and was defined according to the classification of Statistics Netherlands and grouped according to major ethnic groups in the Netherlands and/or similarity in thyroid function tests.[2 3] Maternal smoking status was classified as no smoking, smoking until known pregnancy, and continued smoking during pregnancy. Education level was defined according to total number of education years after primary school and divided into low (≤4), middle (5-10) or high (>10).

Multiple imputation

In the ABCD study, maternal BMI and ethnicity were imputed (missing due to non-response in 11.0% and 1%, respectively). No significant differences in descriptive characteristics were found between the original and imputed datasets.

HAPPY study

Thyroid measurements

In the HAPPY study, TSH, FT4, TPO-Abs, and hCG are measured in Li-heparin plasma using electrochemoluminescence assays (Cobas® e 601, Roche Diagnostics, Mannheim Germany) and the intra- and interassay coefficients of variation were <2.5% for TSH at a range of 0.05-6.0 mU/L, <3.6% for FT4 at a range of 13-33 pmol/L, 7.5% and 14.6% for TPOAbs at 93 IU/L and 25 IU/L and <3.7% for hCG at a range of 5-200 IU/L. TPOAbs were considered positive at >35IU/L.

In the HAPPY study, early pregnancy TSH and FT4 concentrations did not differ between women with, or women without a second measurement (data not shown). As compared to women with both an early pregnancy measurement and a late pregnancy measurement, women with only an early pregnancy measurement had slightly lower early pregnancy hCG (-0.11 SD; *P*=0.048) and were more likely to be TPOAb positive (34/375 (9.1%) *versus* 77/1295 (5.9%), *P*=0.034). Further details in data ascertainment for Generation R and the Happy study have been described previously.[2, 3]

Determinants and covariates

Gestational age at blood sampling was defined using fetal ultrasound data on crown-rump length or biparietal diameter for pregnancy dating.[5] When there was a discrepancy between the calculated term and the ultrasound term, a second ultrasound was used to define the exact gestational age. Information on maternal age, smoking status, maternal education level and obstetrical history was obtained by questionnaires during pregnancy, the Dutch National Perinatal registry and cross checked with medical files. Only Caucasian women with sufficient knowledge of the Dutch language were invited and those who did meet the NHANES criteria were excluded: a known history of previous thyroid dysfunction (Hashimoto thyroiditis or Graves' disease, on hormone replacement therapy or not), a known history of autoimmune disease (e.g., diabetes mellitus, rheumatoid arthritis), and the use of drugs that might interfere with thyroid function (e.g., use of lithium in bipolar patients). [6] Maternal smoking status was classified as no smoking, smoking until known pregnancy (stopped), and continued smoking during pregnancy. Education level was defined according to finished education as none/primary, secondary or higher. Weight and length were measured at intake (same time as blood sample collection) and were used to calculate body mass index (BMI). Information on fertility treatment and gender of the child were obtained from community midwives, obstetricians, and hospital registries. Previous reports showed that this population is iodine sufficient.[7]

Multiple imputation

In the HAPPY study database, maternal late pregnancy BMI, late pregnancy gestational age at blood sampling, early pregnancy BMI and early pregnancy gestational age at blood sampling were imputed (missing due to non-response in 10.3%, 4.2% and <1.0%, respectively).

1. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, et al. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology* 2008;31(4):388-96.

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3. Korevaar TI, Medici M, de Rijke YB, Visser W, de Muinck Keizer-Schrama SM, Jaddoe VW, et al. Ethnic differences in maternal thyroid parameters during pregnancy: the Generation R study. *J Clin Endocrinol Metab* 2013;98(9):3678-86.

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5. Truijens, SM., Meems, M., Kuppens, SM, Broeren MA, Nabbe, KC, Wijnen HA, et al. (2014). The HAPPY study (Holistic Approach to Pregnancy and the first Postpartum Year): Design of a large prospective cohort study. *BMC Pregnancy and Childbirth*, 14, 312.

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7. Kuppens SM, Kooistra L, Wijnen HA, Vader HL, Hasaart TH, Oei SG, Vulsma T, Pop VJ. Neonatal thyroid screening results are related to gestational maternal thyroid function. *Clin Endocrinol (Oxf)* 2011; 75:382–387.

SUPPLEMENTAL TABLE 1. *Characteristics of study population during early pregnancy per cohort.*

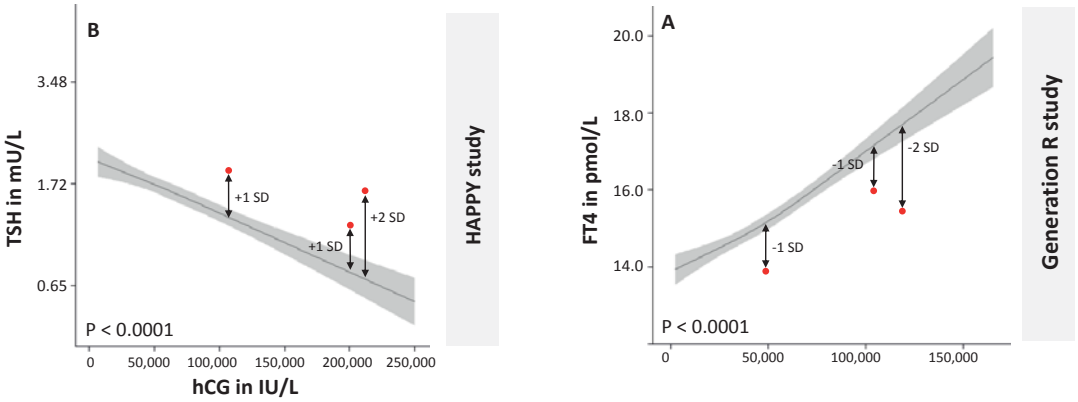
	Generation R		ABCD study		Happy Study	
	Median or N (%)	(95% range)	Median or N (%)	(95% range)	Median or N (%)	(95% range)
Median TSH (mU/L)	1.35	(0.03-4.56)	1.16	(0.12-3.58)	1.45	(0.23-4.43)
Median FT4 (pmol/L)	14.8	(10.3-22.3)	9.5	(7.1-12.7)	14.3	(11.5-18.0)
Gestational age[a]	13.2	(9.6-17.4)	12.9	(8.1-22.9)	13	(11-18)
Maternal age[d]	30.3	(19.5-38.8)	31	(20-39)	30	(23-38)
BMI	23.5	(18.5-35.8)	23.3	(18.7-33.3)	23.1	(18.3-33.7)
Parity[c]						
0	3200	(57.4)	2331	(57.3)	970	(47.2)
1	1653	(29.6)	1270	(31.2)	842	(41.0)
2	525	(9.4)	343	(8.4)	210	(10.2)
>2	201	(3.6)	121	(3.0)	34	(1.7)
Smoking[c]						
Non-smokers	4076	(73.1)	3002	(73.8)	1922	(93.5)
Stopped smokers	499	(8.9)	676	(16.6)		
Smokers	1004	(18.0)	387	(9.5)	134	(6.5)
Education level						
Low	1386	(24.8)	427	(10.5)	112	(5.4)
Middle	2799	(50.2)	1861	(45.8)	647	(31.5)
High	1394	(25.0)	1777	(43.7)	1297	(63.1)
Ethnicity[c]						
Dutch	2803	(51.6)	2209	(56.8)	2007	(97.6)
Moroccan	360	(6.3)	230	(5.4)		
Turkish	474	(8.4)	154	(4.0)		
Surinamese	440	(8.8)	278	(7.2)		
Antillean			63	(1.6)		
Ghanese			49	(1.3)		
Other western	318	(9.0)				
Other non-western	866	(15.9)				
Other			904	(23.3)	49	(2.4)
Child gender[c] (boys %)	2810	(50.4)	1034	(49.6)	1977	(49.6)

[a] At time of blood sampling
[b] Data shown as mean in grams (SD)
[c] Data shown as n (%)
[d] Data shown as median in years

SUPPLEMENTAL TABLE 2. *Risk of premature delivery according to TPOAb cut-offs stratified by TSH cut-offs.*

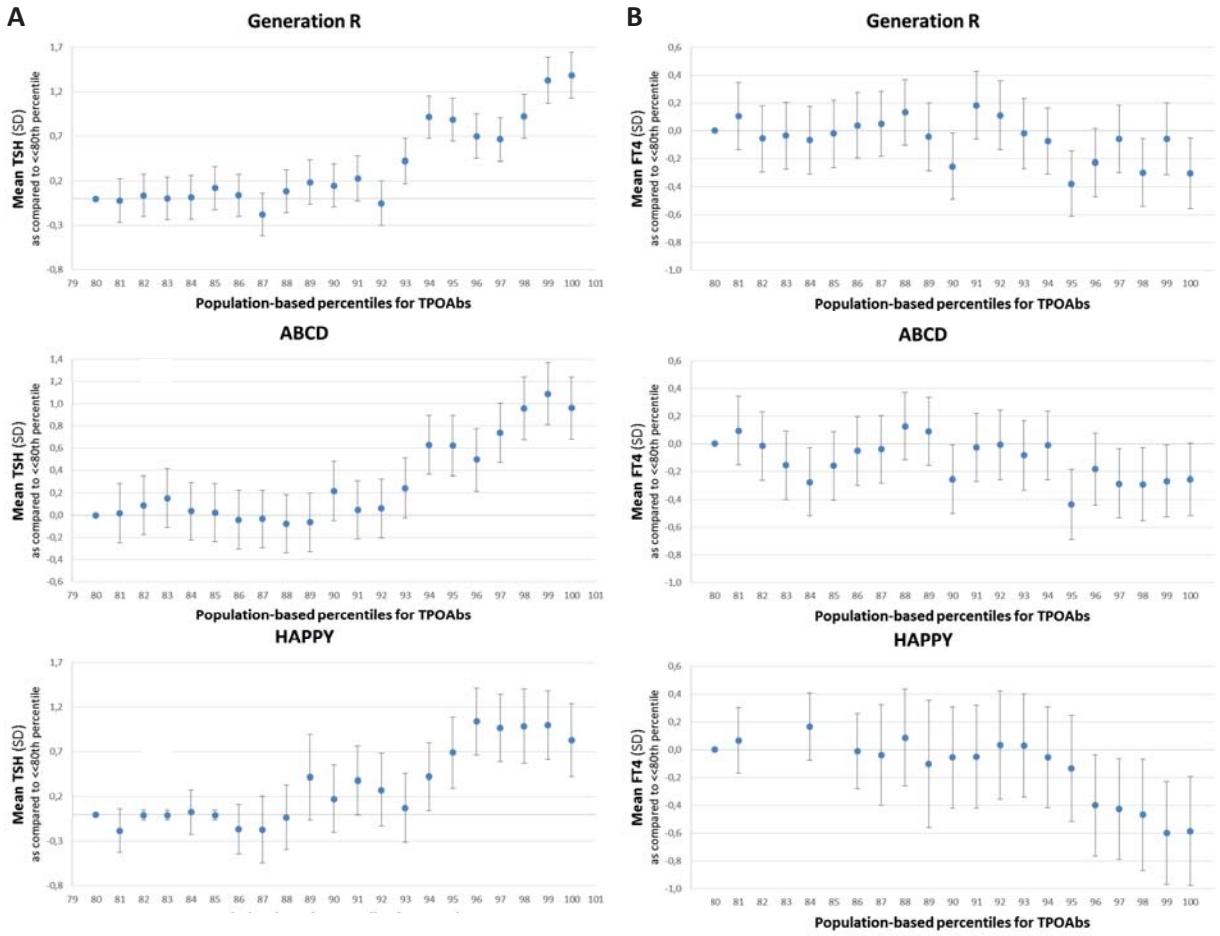
TSH >P95	1.09	1.08	1.36	1.36	1.51	1.65	2.62	2.80	1.08	2.01	2.06	2.00	2.11
TSH >P90	1.05	2.17	2.71	2.85	3.13	2.48	2.86	4.33	2.17	1.73	1.77	1.72	1.85
TSH >P85	1.05	1.71	2.19	2.31	2.70	2.44	2.99	3.79	1.71	1.48	1.51	1.47	1.60
TSH >P80	1.09	1.81	2.27	2.42	2.86	2.92	3.79	2.93	1.81	1.44	1.46	1.43	1.56
TSH >P75	1.08	1.73	2.11	2.31	2.76	2.87	3.39	2.89	1.73	1.43	1.45	1.42	1.49
	TSH only	**p84.5**	**p85.5**	**p86.5**	**p87.5**	**p88.5**	**p89.5**	**p90.5**	**p91.5**	**p92.5**	**p93.5**	**Manufacturer**	**94.5**

SUPPLEMENTAL FIGURE 1. *The association of hCG with FT4 and graphical depiction of deviation from mean.*



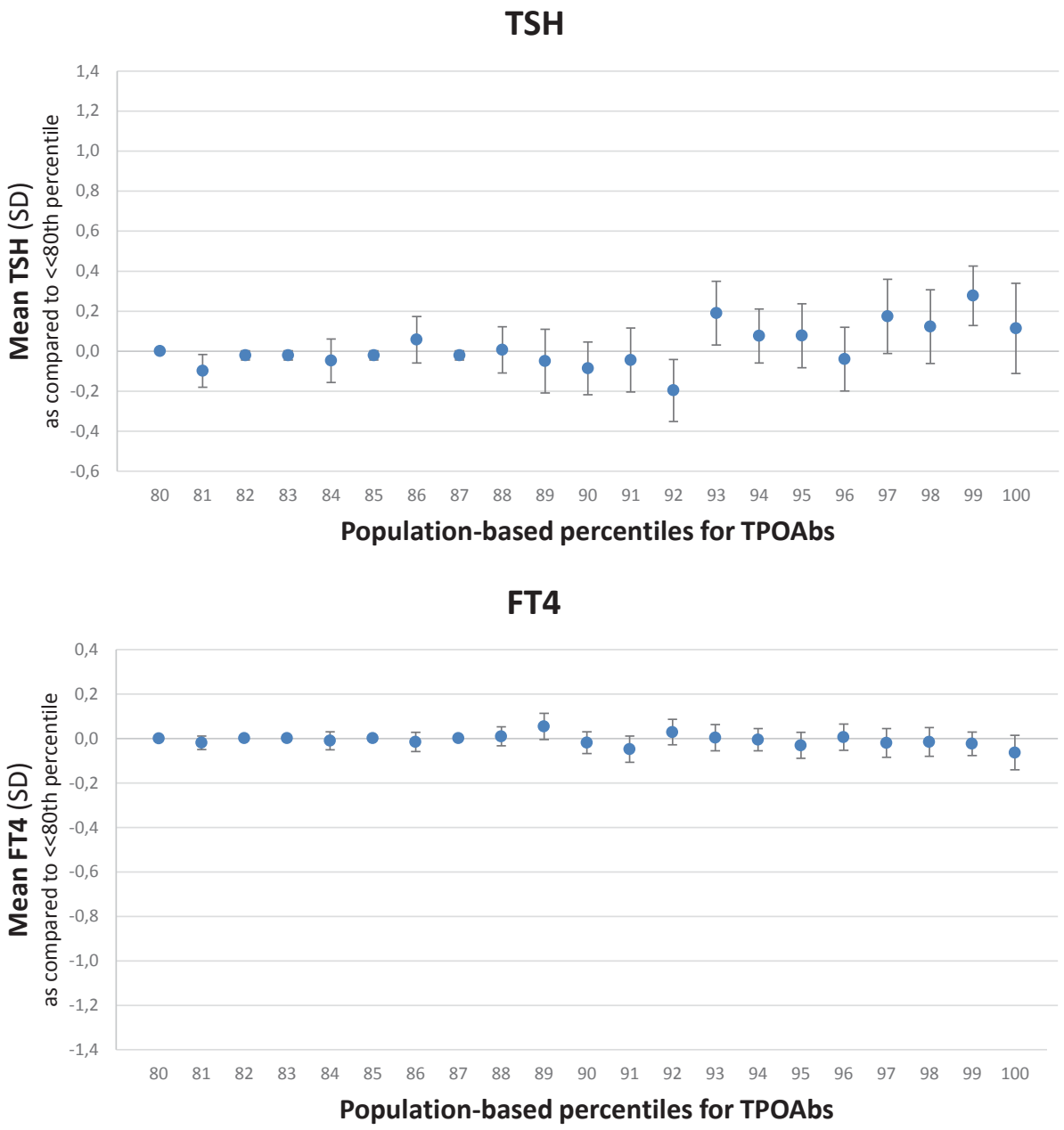
Supplemental Figure 1. shows the association of hCG with FT4 and a graphical depiction of deviation from mean. Figures show the association of hCG with TSH (A) and hCG with FT4 (B) as estimated mean (black line) with 95% confidence interval (grey area). Red dots and black arrows graphically depict the concept of using residuals as a marker for the deviation from the expected association of hCG with TSH or FT4.

SUPPLEMENTAL FIGURE 2. *Population-based TPOAb percentiles and mean TSH (A) and FT4 (B) per cohort.*



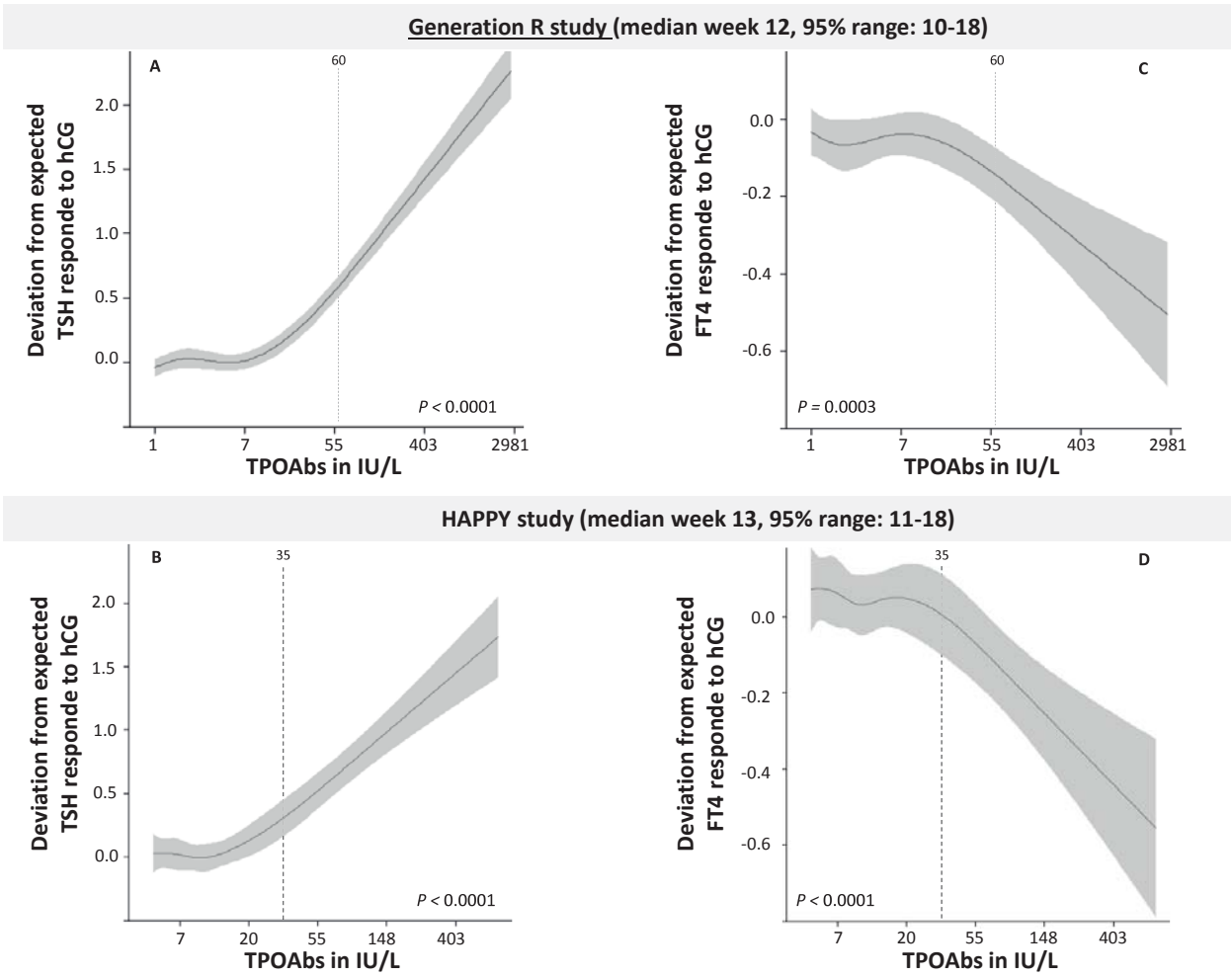
Plots show the mean (±95% CI) difference in TSH (A) and FT4 (B) for each population-based percentile of TPOAbs as compared to the reference group (≤P 80). All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

SUPPLEMENTAL FIGURE 3. *Population-based TPOAb percentiles and mean TSH and FT4 during late pregnancy.*



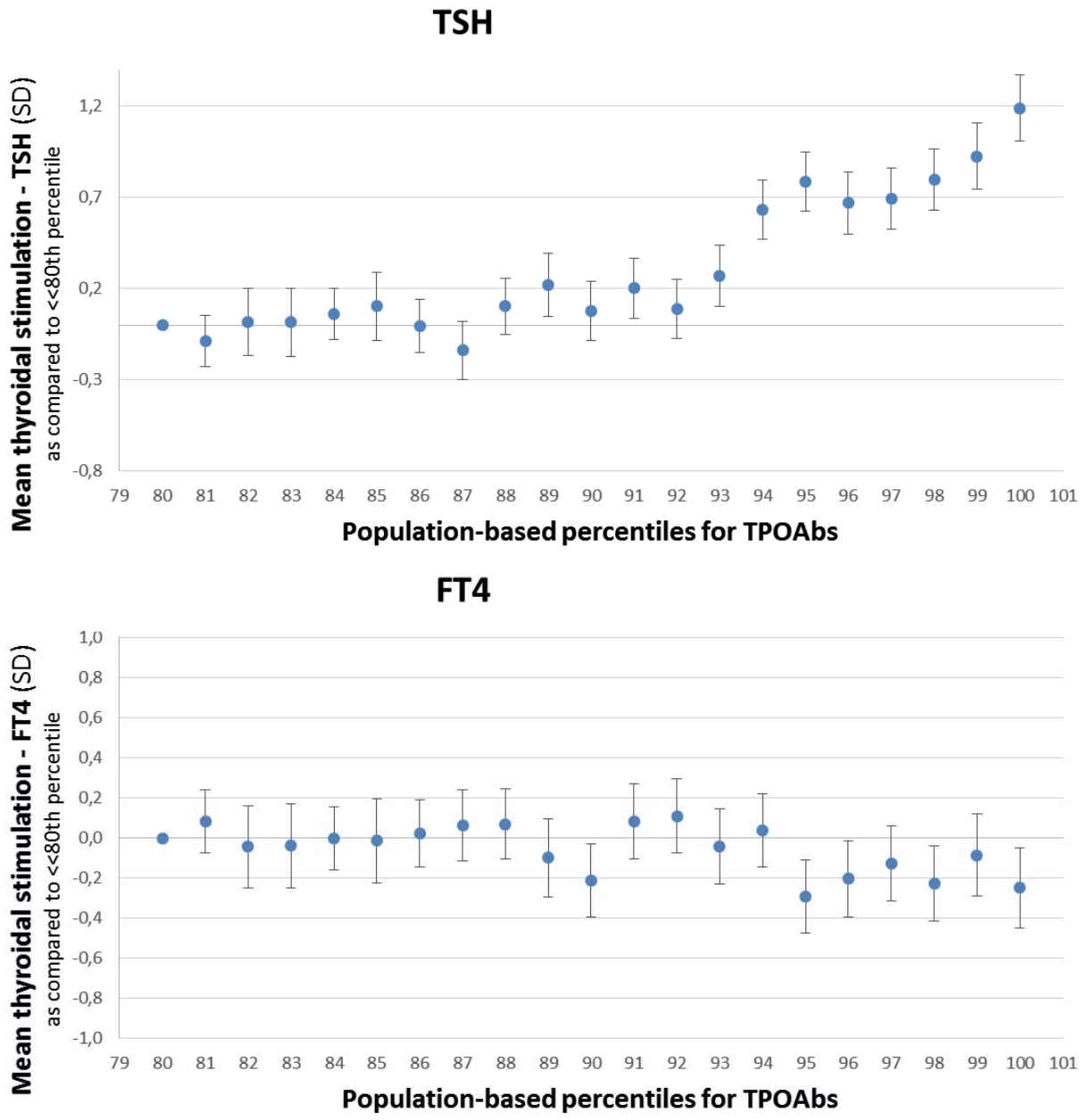
Plots show the mean (±95% CI) difference in TSH and FT4 for each population-based percentile of TPOAbs as compared to the reference group (≤P 80). Group size ranged between 108 and 114. All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

SUPPLEMENTAL FIGURE 4. *The association of TPOAb levels with deviation from mean hCG-mediated changes in TSH and FT4.*



Supplemental Figure 4. shows the association of TPOAb levels and the residuals of the association of hCG with either TSH (A, B) or FT4 (C, D) as estimated mean (black line) with 95% confidence interval (grey area). The vertical lines show currently proposed cut-offs for TPOAb positivity (35, 60 or 100 IU/L). All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

SUPPLEMENTAL FIGURE 5. *Population-based TPOAb percentiles and deviation from mean thyroidal response to hCG.*



Plots show the mean (±95% CI) difference in TSH and FT4 for each population-based percentile of TPOAbs as compared to the reference group (≤P 80). All analyses have been adjusted for gestational age at blood sampling, maternal smoking, parity, BMI, age and ethnicity.

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CHAPTER 14

MATERNAL TOTAL T4 DURING THE FIRST HALF OF PREGNANCY: PHYSIOLOGIC ASPECTS AND THE RISK OF ADVERSE OUTCOMES IN COMPARISON TO FREE T4

Tim I.M. Korevaar, Layal Chaker, Marco Medici, Yolanda B. de Rijke, Vincent W.V. Jaddoe, Eric A.P. Steegers, Henning Tiemeier, Theo J. Visser and Robin P. Peeters

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ABSTRACT

BACKGROUND Total T4 (TT4) has been suggested as a marker for maternal thyroid function during pregnancy because as compared to FT4 1) TT4 measurement is not affected by binding protein interference, 2) TT4 is considered to be more stable from the second trimester onwards and 3) TT4 better reflects changes in the hypothalamic-pituitary-thyroid axis. However, this is based on data from small studies and, more importantly, it is unknown whether TT4 is associated with adverse pregnancy or child outcomes.

AIM We aimed to investigate TT4 physiological aspects and associations with clinical endpoints.

METHODS We selected 5647 mother-child pairs from a large population-based prospective cohort with data on maternal TSH, FT4 and TT4 during early pregnancy (median 13.2wks, 95% range 9.8-17.6). We used multivariable (non)linear and logistic regression models to study the association of maternal TT4 with preeclampsia, premature delivery, birth weight and offspring IQ and compare the results with previously obtained results for FT4.

RESULTS The change of mean TT4 levels was 27.5% compared to 20.2% for FT4. There was a loglinear association of TT4 and FT4 with TSH, but the explained variability of TSH was much lower for TT4 than for FT4 (R-squared TT4: 2.5% versus 8.0% for FT4). In contrast to FT4, there was no independent association of maternal TT4 with preeclampsia, premature delivery, birth weight or offspring IQ.

CONCLUSION Maternal TT4 levels are highly variable in the first half of pregnancy and are poorly related to maternal TSH. This study shows that maternal TT4 levels are either not associated, or not better associated as compared to FT4, with adverse pregnancy or child outcomes. This suggests that the maternal TT4 is inferior to FT4 in the assessment of maternal thyroid function during the first half of pregnancy.

INTRODUCTION

Adequate thyroid hormone availability during early pregnancy is essential for a normal pregnancy outcome as well as proper fetal growth and development. Gestational thyroid dysfunction has been associated with an increased risk of adverse clinical outcomes, including preeclampsia, premature delivery, adverse effects on birth weight and cognitive development of the child.[1-4] The majority of physicians use both TSH and FT4 for the assessment of maternal thyroid function during pregnancy.[5-8] However, several immunoassays may not adequately measure FT4 levels due to the high protein binding state that is present during pregnancy, particularly during late pregnancy.[9] This is one of the main reasons that current guidelines of the American Thyroid Association (ATA), the Endocrine Society (ES) and the European Thyroid Association (ETA) suggest TT4 as an alternative measure for maternal thyroid function during pregnancy.[10-12]

Apart from the fact that TT4 assays are less affected by differences in binding proteins, various other (physiological) arguments may favor the use of TT4 over FT4. First of all, TT4 levels are reported to be more stable throughout pregnancy compared to FT4 levels.[9-11] Secondly, TT4 levels may have a better log-linear relationship with maternal TSH than FT4, thus better reflecting changes in the hypothalamic-pituitary-thyroid axis (HPT-axis).[9-11] A counter argument against using TT4 as a measurement of gestational thyroid function is that TT4 practically reflects the concentrations of biologically inactive hormone since more than 99% of the TT4 is protein-bound. Furthermore, it may not be clinically feasible to use TT4 as a measure if maternal thyroid hormone status as most gestational thyroid function tests are performed during the first half of pregnancy, when there is a rapid increase in thyroxine binding globulin (TBG) leading to a high variability in TT4 levels.[13]

Interestingly, all arguments in the discussion of the usefulness of TT4 during pregnancy are based on physiological assumptions and not on the association of TT4 with adverse pregnancy and child outcomes. Although many studies have investigated the effects of abnormal maternal TSH and/or FT4 levels, there are no data on the association of maternal TT4 with adverse pregnancy or child outcomes. The main aim of this study was therefore to investigate physiological characteristics of TT4, to assess the extent of overlap between women classified as having low or high levels of TT4 and FT4 and to study the association of TT4 with adverse pregnancy and child outcomes.

METHODS

Design and population

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[14] Of the 7069 women that were enrolled during early pregnancy (95% range 8-18th week of pregnancy; considered as generalizable range) TT4 levels were determined in a total of 5940 women for which serum was available. Women without data available for TT4 were on average older (+0.3 years; P=0.015), had a lower BMI (-0.98 points; P<0.001) and were more likely to have a high education level (OR 1.16; P=0.007) suggesting a slight selection bias favoring the inclusion of women with a higher socioeconomic status. After exclusion of women with twin pregnancies (N=128), preexisting thyroid disease or thyroid interfering medication usage (N=89) and women with fertility treatment (N=76) the final study population included 5647 women. In these women we studied the physiological characteristics of TT4 namely, the association of gestational age with TT4, the associations of FT4 and TT4 with TSH, and we also assessed the overlap of women within groups of low/high FT4 and TT4 (group numbers may differ slightly from group numbers for cut-offs in

papers on clinical outcomes due to the use or non-use of imputation for FT4 levels). In order to study the associations of TT4 with adverse clinical outcomes we revisited previously studied associations using the same databases (for optimal comparison with FT4) between maternal thyroid function and preeclampsia (TT4 available for 5136 out of initial 5153 subject with FT4 and outcome data available), premature delivery and birth weight (TT4 available for 5641 out of initial 5971) and offspring IQ (TT4 available for 3636 out of initial 3839).[3,15,16] The overlap between availability for FT4 and TT4 was not 100% due to random measurement of error and/or lack of adequate serum availability for FT4 or TT4 measurements in the lab.

Thyroid measurements

Maternal serum samples were obtained in early pregnancy (mean 13.5 weeks; SD 2.0). Plain tubes were centrifuged and serum was stored at -80 C. TSH, FT4 and TT4 were determined in maternal serum samples using chemiluminescence assays (Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). The intra- and interassay coefficients of variation were <4.1% for TSH, <5.4% for FT4, and <6.4% for TT4.[17]

Clinical outcomes

The definition of all outcomes used for this study have previously been described in detail.[3,15,16] In short, non-verbal child IQ was assessed using two subtests of a well-validated Dutch nonverbal intelligence test, the 'Snijders-Oomen niet-verbale intelligentie test' (median age of six years (95% range 5.6 – 7.9 years). This test broadly assesses the spectrum of intelligence functions without depending upon language skills and is therefore appropriate for the assessment of cognitive abilities of ethnic minorities and/or children with problems with verbal communication. The two subsets were mosaics, which assesses spatial visualization abilities, and categories, which assesses abstract reasoning abilities (correlation with complete test r = 0.86). Raw test scores were converted into nonverbal IQ scores using normal values tailored to exact age. Information on birth weight and gestational age at birth was obtained from community midwives, obstetricians and hospital registries. Birth weight was assessed using standard deviations scores, adjusted to gestational age at birth, scores were constructed using the Niklasson percentile growth curves. Prematurity was defined as a gestational age at birth <37 weeks or <34 weeks (very premature delivery). Certified medical doctors reviewed women's hospital charts and defined preeclampsia according to the criteria of the International Society for the Study of Hypertension in Pregnancy. Preeclampsia was identified as the development of a systolic BP of 140 mm Hg or greater and/or a diastolic BP of 90 mm Hg or greater (at least two BP readings) after 20 weeks of gestation in a previously normotensive woman, plus the presence of proteinuria (defined as two or more dipstick readings of 2 or greater, one catheter sample reading of 1or greater, or a 24 h urine collection containing at least 300 mg of protein).

Statistical analyses

We used multivariable linear regression models to study the association of FT4 with TT4, 2) the association of gestational age at blood sampling with TT4, 3) the association of FT4 and TT4 with TSH and 4) the association of FT4 and TT4 with child IQ and birth weight. The association of FT4 and TT4 with premature delivery and preeclampsia was investigated using multivariable logistic regression models. We assessed potential non-linearity in linear and logistic regression models by using restricted cubic splines utilizing three knots. We tested for differences in the association of FT4 or TT4 with TSH according to gestational age by introducing a product interaction term of gestational age with FT4 or TT4 to the model, and subsequently stratified analyses in case of a P-value for the interaction term of <0.15. For covariates with missing data (0% for gestational age at blood sampling, child gender and maternal

age, <1.0% for maternal BMI and parity, 4.0% for maternal ethnicity, 7.1% for maternal education and 13.0% for maternal smoking) imputation according to the Markov Chain Monte Carlo method was used. For comparability, all analyses on TT4 and FT4 were adjusted for the covariates described in the previous publications on FT4[3,15,16]; all models were at least adjusted for maternal age, smoking, parity, BMI, gestational age at blood sampling and fetal gender.

RESULTS

Descriptive statistics for the study population are shown in Supplemental Table 1. TT4 was positively association with FT4, and TT4 explained 30.0% of the variability in FT4 (Supplemental Figure 1). Gestational age at blood sampling was positively associated with maternal TT4 and the difference in mean TT4 levels between the 8th to 18th week of pregnancy was 27.5% (Figure 1A). Gestational age at blood sampling was negatively associated with maternal FT4 and the difference in mean FT4 between the 8th to 18th week of pregnancy was approximately 20.2% (Figure 1B). Likewise, there was a negative, loglinear association of maternal TT4 with TSH and maternal TT4 explained 2.5% of the variability in TSH (Figure 1C). There was a negative, loglinear association of maternal FT4 with TSH and maternal FT4 explained 8.0% in the variability of TSH (Figure 1D). The loglinear association of both TT4 and FT4 with TSH attenuated with a higher gestational age at blood measurement (both *P* interaction<0.001). The extent by which the association attenuated was similar for TT4 as for FT4 (Supplemental Figure 2). Of all women with a low TT4 according to various percentile cut-offs (2.5th, 5th and 10th percentile), 17.7% to 26.7% also had a low FT4 (Supplemental Table 2). Of all women with a high TT4 according to various percentile cut-offs (97.5th, 95th and 90th percentile), 34.8% to 37.6% also had high FT4 levels (Supplemental Table 2).

Adverse clinical outcomes

Women within the highest quintile of the FT4 normal range had a 2.1-fold increased risk of preeclampsia (Table 1). In contrast, women within the highest quintile of the TT4 normal range did not have an increased risk of preeclampsia (Table 1). Women with a low FT4 had a 2.5 and 3.9-fold increased risk for premature and very premature delivery, respectively (Table 2). Women with a low TT4 did not have an increased risk of premature delivery or very premature delivery (Table 2).

There was a negative linear association between maternal TT4 and child birth weight (Figure 2A) which did not persist after additional correction for FT4 (Figure 2B; association of FT4 with birth weight remained similar, see Supplemental Figure 3A). There was a negative linear association between maternal TT4 and child IQ (Supplemental Figure 4A). This association persisted after additional correction for maternal FT4 levels (Supplemental Figure 4B; association of FT4 with child IQ remained similar, Supplemental Figure 3B), therefore we additionally investigated the added value of both TT4 and FT4 levels in the identification of pregnancies associated with a lower IQ of the offspring. Women with a low gestational FT4 had offspring with lower IQ, irrespective of the presence of low TT4 (Table 3). Women with only low TT4, but normal FT4, did not have offspring with a lower IQ as compared to the reference group (Table 3). Women with a high gestational FT4 had offspring with a lower IQ, irrespective of the presence of high TT4 (Table 3). Women with only high TT4, but normal FT4, did not have offspring with a lower IQ as compared to the reference group (Table 3). Similar results were obtained with 2.5th and 10th percentile cut-offs (data not shown).

FIGURE 1. *Physiological characteristics of maternal total T4 and free T4 during early pregnancy.*

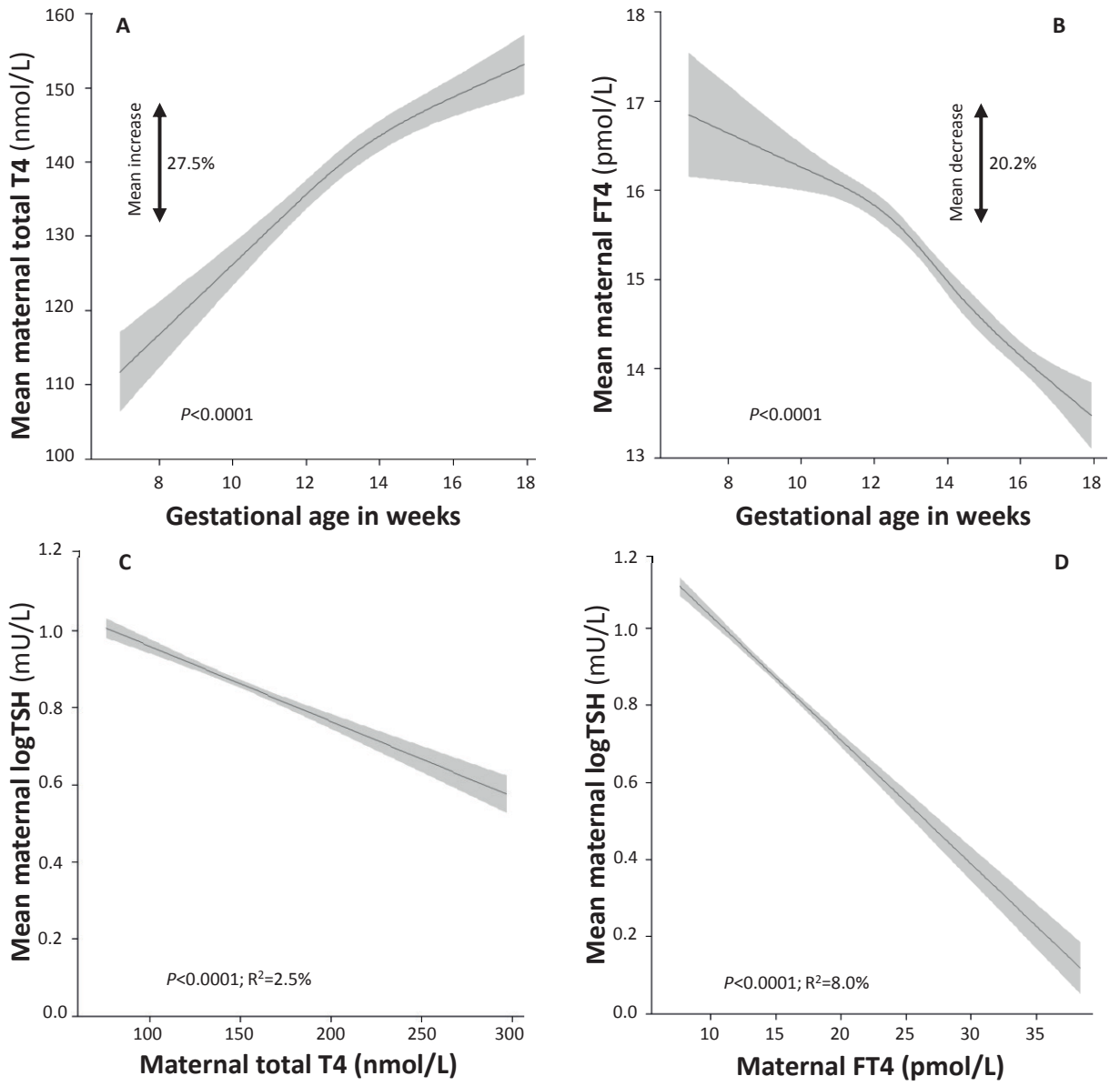


FIGURE 2. *The association of maternal total T4 with birth weight.*

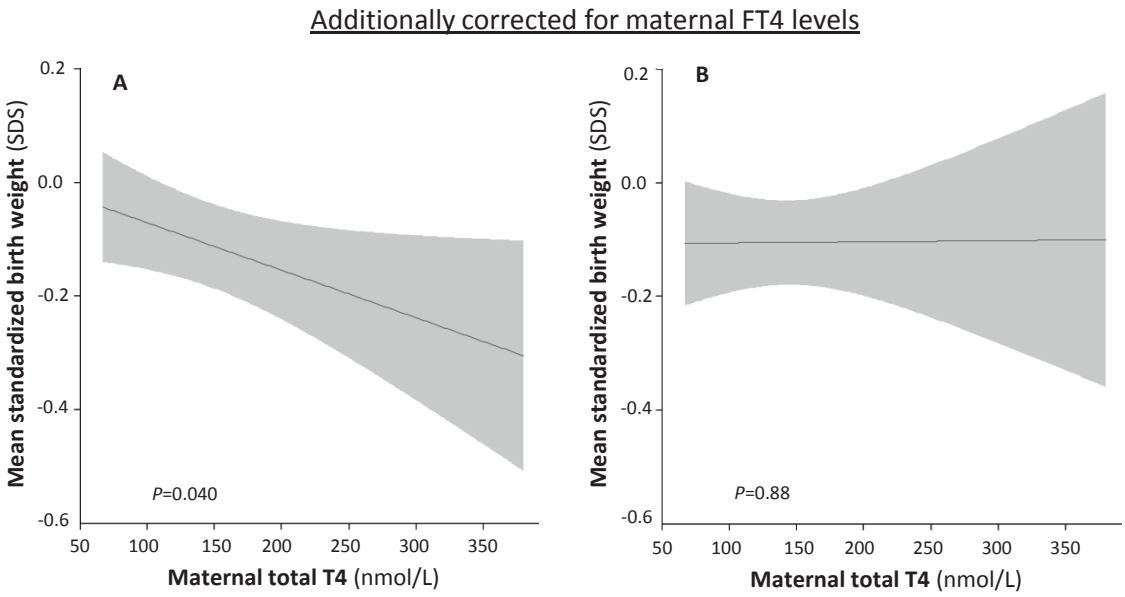


TABLE 1. *Association of decreased FT4 or TT4 with the risk of preeclampsia.*

FT4 quintiles (total N; % preeclampsia)		OR (95%CI)	*P*
1st quintile	(N=862; 2.8%)	1.68 (0.85-3.34)	0.14
2nd quintile	(N=915; 2.7%)	1.68 (0.85-3.31)	0.14
3rd quintile	(N=974; 1.5%)	ref	ref
4th quintile	(N=897; 2.5%)	1.81 (0.90-3.65)	0.10
5th quintile	(N=879; 3.0%)	2.06 (1.04-4.08)	0.04
TT4 quintiles (total N; % preeclampsia)		**OR (95%CI)**	***P***
1st quintile	(N=955; 1.7%)	0.68 (0.35-1.29)	0.24
2nd quintile	(N=864; 2.5%)	1.05 (0.58-1.91)	0.86
3rd quintile	(N=932; 2.6%)	ref	ref
4th quintile	(N=887; 2.8%)	1.10 (0.62-1.96)	0.74
5th quintile	(N=889; 2.9%)	0.99 (0.56-1.78)	0.99

TABLE 2. *Association of decreased FT4 or TT4 with the risk of premature delivery.*

		OR (95%CI)	P
Premature delivery (<37 weeks)			
Decreased FT4	(N=146)	2.48 (1.42-4.32)	0.001
Euthyroid	(N=5037)	ref	ref
Premature delivery (<34 weeks)			
Decreased FT4	(N=146)	3.90 (1.76-8.68)	0.001
Euthyroid	(N=5037)	ref	ref
Premature delivery (<37 weeks)			
Decreased TT4	(N=141)	0.68 (0.27-1.69)	0.41
Euthyroid	(N=5037)	ref	ref
Premature delivery (<34 weeks)			
Decreased TT4	(N=141)	0.95 (0.23-4.02)	0.95
Euthyroid	(N=5037)	ref	ref

TABLE 3. *Association of FT4, TT4 and overlapping with offspring IQ.*

		IQ at median age 6 years		
		Mean ±SE		P
Low FT4 and/or TT4				
Normal FT4 & TT4	(N=3224)	101.9	±0.2	ref
Only low FT4	(N=137)	98.6	±1.2	0.007
Only low TT4	(N=121)	101.8	±1.3	0.77
Low FT4 & TT4	(N=62)	97.8	±2.2	0.06
High FT4 and/or TT4				
Normal FT4 & TT4	(N=3224)	102.0	±0.2	ref
Only high FT4	(N=142)	99.1	±1.3	0.02
Only high TT4	(N=139)	100.8	±1.3	0.40
High FT4 & TT4	(N=52)	98.2	±1.8	0.04

Low FT4 and low TT4 were defined as FT4/TT4 below the 5th percentile based on previous observations (ref 2).

DISCUSSION

In this large population-based prospective study, we demonstrate that the relative variability in TT4 in the first half of pregnancy is larger as compared to maternal FT4 levels. We also show that there is a loglinear association of TT4 levels with TSH which explained a lower proportion of the variability in TSH (2.5%) as compared to FT4 (8.0%). Finally, we show that, in contrast to FT4, there is no association of TT4 with any of the main thyroid hormone related adverse pregnancy or child outcomes.

Absolute values of FT4 may be under or overestimated when measured by immunoassays during pregnancy, particularly in the third trimester.[9,18,19] However, the diagnosis of maternal thyroid dysfunction is most relevant during early pregnancy when the developing fetus is dependent on the production of maternal thyroid hormones. Based on an overview of ten small studies (median N=50, range 8-606) showing that the mean maternal TT4 in a population is relatively stable over the three trimesters,[9] it has been proposed that TT4 can be used to assess thyroid (dys)function during pregnancy.[9-11] However, since the rise in TBG and other changes in thyroid hormone binding proteins is continuous over time and does not change per trimester, these data should most preferably be analysed continuously instead of categorized into three trimesters. In the current study, we show that the physiological rise in TT4 occurs from at least the 8th to 18th week of pregnancy and that this change is 37% higher as compared to FT4. This indicates that TT4 is not as stable in the first half of pregnancy as previously suggested based on analyses focusing on mean trimester values. The HPT-axis is reflected by a loglinear relationship between thyroid hormones and TSH.[20-23] In the current study we demonstrate that TT4 does not properly reflect changes in the hypothalamic-pituitary-thyroid axis as the expected loglinear relationship with TSH was poor (explained variability of 2.5% versus 8.0% for FT4). The gestational change in combination with the poorer reflection of the HPT-axis for TT4 as compared to FT4 are additional arguments that FT4 should be the preferred measurement during of the first half of pregnancy.

The most important argument for the use of maternal TT4 in the assessment of gestational thyroid function would be the association of TT4 with adverse pregnancy and/or offspring outcomes. Large studies on thyroid dysfunction, as defined by TSH and/or FT4, have shown an association of thyroid dysfunction with an increased risk of premature delivery, abnormal birth weight and offspring IQ. To our knowledge, there are no data on the association of maternal TT4 with adverse pregnancy or child outcomes. In this study, we demonstrate that TT4 is inferior to FT4 in the assessment of maternal

thyroid function during pregnancy due to the poor associations of TT4 with the main thyroid hormone related pregnancy and child outcomes.

There is a very strong association of maternal TT4 with FT4. However, we demonstrate that of the women with low TT4, only 17.7-26.7% also had low FT4. For women with high TT4, the overlap with high FT4 was 33.5-37.6%. The small overlap of women with abnormal TT4 levels and women with abnormal FT4 levels may be explained by inter-individual differences in binding proteins or their capacity. In addition to inter-individual differences in common genetic variants of the tranthyretin and TBG gene, differences in exposure to endocrine disrupting compounds (EDCs) may also contribute to the relatively large differences in overlap between abnormal TT4 and FT4 levels observed in our study.[24-26] There is a rapid increase in human exposure to EDCs[27] and many EDCs have been shown to be potent competitive binders to thyroid hormone binding protein such as transthyretin and TBG.[28,29]

To our knowledge, this is the first study that systematically investigated the feasibility of the use of TT4 as a marker of maternal thyroid function during the first half pregnancy. We were able to study physiological aspects of maternal TT4 and its association with several adverse outcomes in a large population-based sample. Furthermore, we had detailed phenotype data available which also included FT4 levels measured in the same sample. Our study was limited by the fact that FT4 was measured by a single immunoassay/method. This makes our estimates on the comparison of TT4 with FT4 less generalizable to centers where a different assay is used. However, although studies have shown that absolute levels of FT4 may differ between immunoassays, it is important to realize that the inter individual differences are similar as all immunoassays have a high correlation with LC/MS and each other.[18,19,30,31] This means that if a method specific reference range is used, immunoassays will correctly identify women with a low or high FT4. This approach, which is also advocated in the ATA, ES and ETA guidelines, will increase generalizability of our results.[11] We were also limited by the availability of a single FT4 and TT4 measurement and we were therefore unable to assess the intra-individual changes throughout gestation. However, a single measurement does mimic clinical practice when the patient risk assessment and treatment indication is frequently determined after the first measurement. Finally, we did not measure TBG, transthyretin or albumin and we were therefore not able to further investigate the underlying physiological mechanisms behind the low overlap between the patients with low or high TT4 versus FT4.

In conclusion, maternal TT4 levels during pregnancy have considerable variability in the first half of pregnancy, maternal TT4 levels are a poor reflection of the hypothalamic-pituitary-thyroid axis and maternal TT4 levels are not meaningfully associated with any adverse pregnancy or child outcomes. This suggests that the there is no (added) value of maternal TT4 in the assessment of maternal thyroid function during the first half of pregnancy over TSH and FT4 measurements. In addition, the lack of association of TT4 with any adverse pregnancy outcomes suggests that there is no role for TT4 measurement in gestational thyroid disease screening modalities. Future studies are needed to investigate to what extent differences in common genetic variants, inter-individual EDC levels and/or differences in binding protein ratios may underlie the indiscriminating character of maternal TT4 as a marker for thyroid function during pregnancy.

APPENDIX

SUPPLEMENTAL TABLE 1 *Descriptive statistics of 5647 women from the Generation R cohort.*

		Median	(95% range)
TPOAb positivity[c]			
TSH	(mU/L)	1.34	(0.04 - 4.46)
FT4	(pmol/L)	14.8	(10.3 - 22.3)
Total T4	(nmol/L)	145	(95 - 221)
TPOAb positivity[c]		298	(5.3)
Gestational age[a]		13.2	(9.6 - 17.6)
Maternal age[d]		30.3	(19.4 – 38.9)
BMI		23.5	(18.5 - 35.6)
Parity[c]			
0		3235	(57.3)
1		1680	(29.8)
2		522	(9.3)
>2		210	(3.7)
Smoking[c,e]			
Non-smokers		4146	(73.4)
Stopped smokers		509	(9.0)
Smokers		992	(17.6)
Education level[e]			
None/Low		591	(10.5)
Middle		2602	(46.1)
High		2454	(43.5)
Ethnicity[c,e]			
Dutch		2900	(51.4)
Moroccan		346	(6.1)
Surinamese		482	(8.5)
Turkish		464	(8.2)
African		316	(5.6)
European		467	(8.3)
Other western		567	(10.0)
Other non-western		105	(1.9)
Fetal sex[c] (boys %)		2849	(50.5)

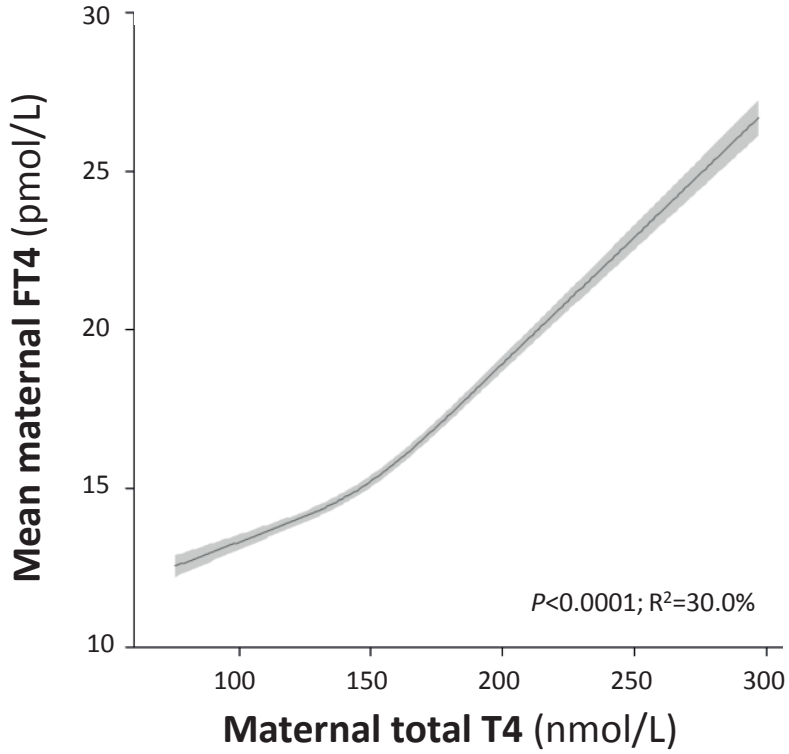
[a] At time of blood sampling; data shown as median in weeks
[b] Data shown as mean in (SD)
[c] Data shown as n (%)
[d] Data shown as median in years
[e] Data shown after imputation of missing data (see methods).
Missing data on covariates was 0% for gestational age at blood sampling, child gender and maternal age, <1.0% for maternal BMI and parity, 4.0% for maternal ethnicity, to 7.1% for maternal education and 13.0% for maternal smoking and numbers are shown after multiple imputation (see methods section).

SUPPLEMENTAL TABLE 2 *Overlap of women with low or high gestational TT4 and FT4.*

Cut-off value*	TT4 (N)	FT4 (N)	Overlapping (N(%))
<2.5th percentile	141	139	25 (17.7%)
<5th percentile	277	275	67 (24.2%)
<10th percentile	546	556	146 (26.7%)
>97.5th percentile	138	146	48 (34.8%)
>95th percentile	281	289	94 (33.5%)
>90th percentile	556	550	209 (37.6%)

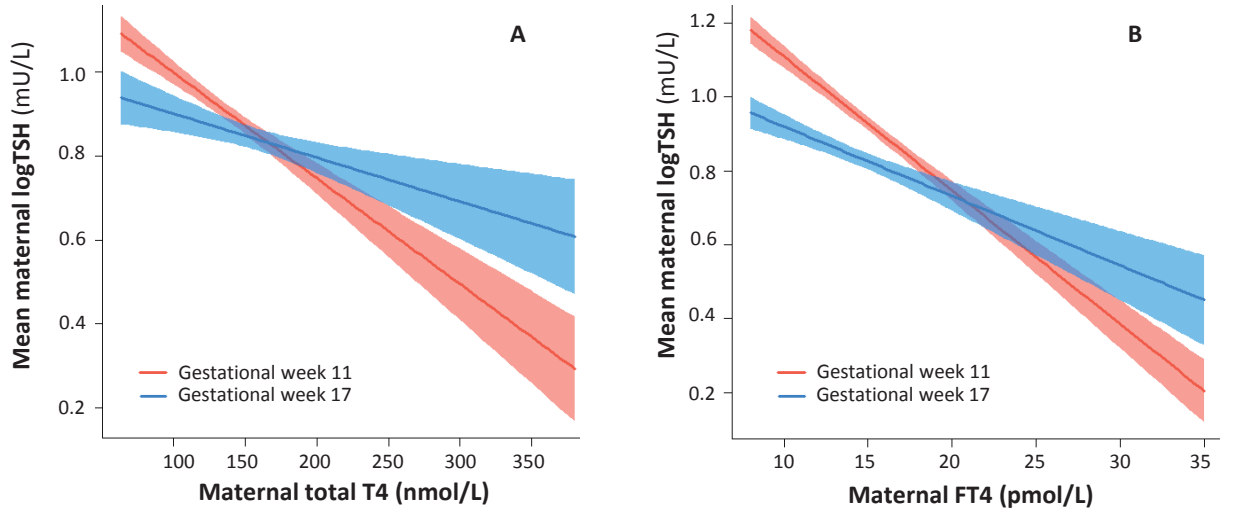
* Unequal numbers for TT4 and FT4 due to dissimilarities in number of individuals with the same FT4/TT4 percentile cut-off level.

SUPPLEMENTAL FIGURE 1. *The association of maternal total T4 with FT4.*



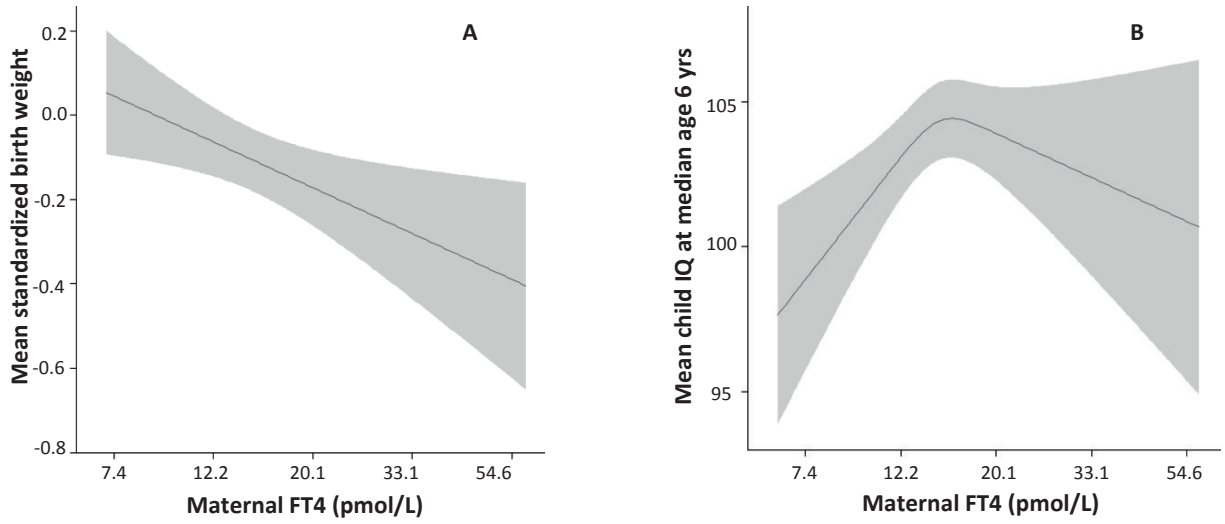
Supplemental Figure shows the association of maternal total T4 levels with maternal FT4 levels during pregnancy as predicted mean (black line) with 95 percent confidence interval (grey area). Median and interquartile range for total T4 were 145 (126-167).

SUPPLEMENTAL FIGURE 2. *Differences in the loglinear association of maternal free T4 or total T4 with TSH according to gestational age at blood sampling.*



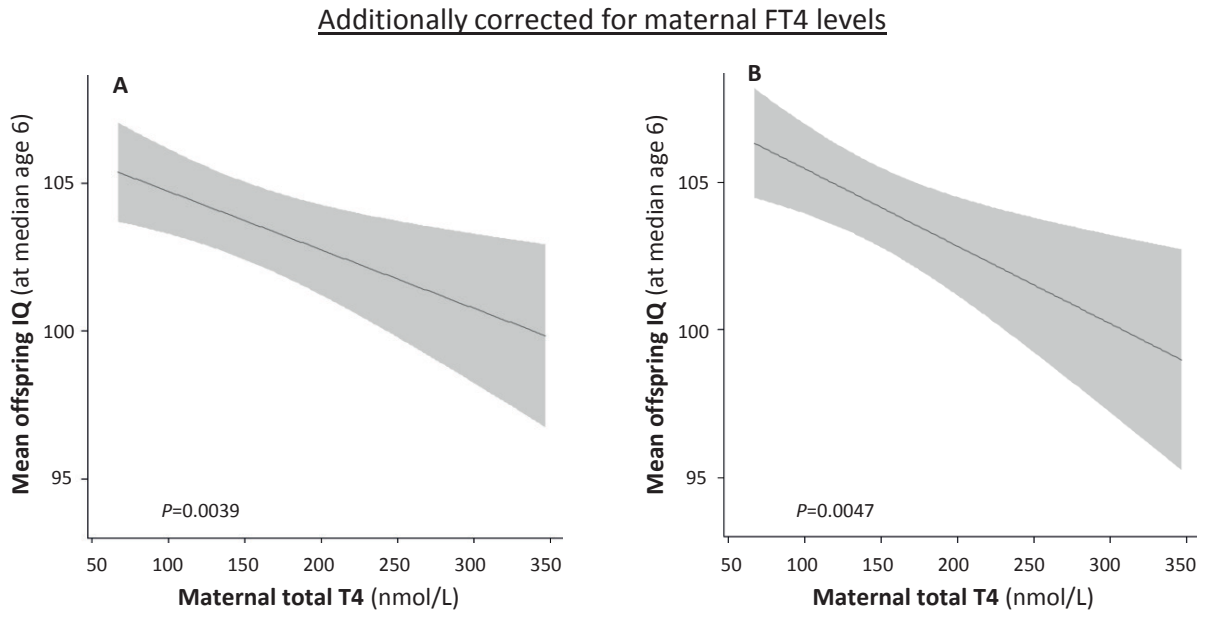
Supplemental Figure shows the association of maternal total T4 levels (A) and FT4 levels (B) with TSH during pregnancy stratified for gestational age at blood sampling week 11 (red) and 17 (blue) as predicted mean (line) with 95 percent confidence interval (surrounding area). Median and interquartile range for total T4 were 145 (126-167) and for FT4 were 14.8 (13.0-15.2).

SUPPLEMENTAL FIGURE 3. *The association of maternal FT4 with child birth weight and IQ after additional correction for maternal TT4.*



Supplemental Figure shows the association of maternal total FT4 levels during pregnancy with standardized offspring birth weight (A) and offspring IQ at age 6 (B) for comparison of TT4 analyses with previously published data on FT4 as predicted mean (black line) with 95 percent confidence interval (grey area). Median and interquartile range for total T4 were 145 (126-167).

SUPPLEMENTAL FIGURE 4. *The association of maternal total T4 with offspring IQ.*



Supplemental Figure shows the association of maternal total TT4 levels during pregnancy with offspring offspring IQ at age 6 (B) and additionally adjusted for maternal FT4 (B) as predicted mean (black line) with 95 percent confidence interval (grey area). Median and interquartile range for total T4 were 145 (126-167).

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CHAPTER 15

THYROID AUTOIMMUNITY IMPAIRS THE THYROIDAL RESPONSE TO HCG: TWO POPULATION-BASED PROSPECTIVE COHORT STUDIES

Tim I.M. Korevaar, Eric A.P. Steegers, Victor J. Pop, Maarten Broere, Layal Chaker, Yolanda B. de Rijke, Vincent W.V. Jaddoe, Marco Medici, Theo J. Visser, Henning Tiemeier and Robin P. Peeters

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ABSTRACT

CONTEXT Thyroperoxidase antibody (TPOAb) positivity is the main risk factor for thyroid dysfunction during pregnancy and is consistently associated with premature delivery. However, the underlying mechanism is currently unknown. We hypothesized that TPOAb positivity may interfere with gestational thyroid stimulation induced by the pregnancy hormone human chorionic gonadotropin (hCG).

DESIGN, SETTING AND PARTICIPANTS TSH, FT4, TPOAbs and/or hCG concentrations were measured in early and late pregnancy of 7587 pregnant women from two Dutch population-based prospective cohorts (N=5924 Generation R; N=1663 HAPPY).

INTERVENTIONS None.

MAIN OUTCOME MEASURE(S) Thyroidal response to hCG stimulation, premature delivery.

RESULTS In TPOAb negative women, hCG was positively associated with FT4 and negatively with TSH in both cohorts ($P<0.0001$). In contrast, in TPOAb positive women, hCG was not associated with FT4 or TSH in both cohorts (all $P>0.40$; P-interaction TPOAb positive vs negative ≤ 0.05).

Overall, TPOAb positivity was associated with a 1.7-fold higher risk of premature delivery. TPOAb positive women with an adequate response of FT4 to hCG (high FT4 with high hCG) did not have a higher risk of premature delivery. In contrast, TPOAb positive women with an inadequate FT4 response to hCG (low FT4 with high hCG) had a 2.2 to 2.8-fold higher risk of premature delivery.

CONCLUSION TPOAb positive women display an impaired thyroidal response to hCG and this may explain the higher risk of premature delivery in these women. This abnormal response in TPOAb positive women might suggest that these women require a different treatment approach than TPOAb negative women.

INTRODUCTION

Maternal thyroid dysfunction occurs in 5-18% of all pregnancies and is associated with a higher risk of various adverse pregnancy outcomes including premature delivery.[1-4] During pregnancy there is an increased demand for thyroid hormone. This is met via stimulation of the thyroid by the pregnancy hormone human chorionic gonadotropin (hCG), which stimulates the thyroid via its affinity for the TSH receptor.[5] The rapid increase of hCG during early pregnancy results in an increase in serum free thyroxine (FT4) and a subsequent decrease in TSH concentrations as compared to a non-pregnancy state.[5]

Thyroperoxidase antibody (TPOAb) positivity, which reflects thyroid autoimmunity, is the most important risk factor for thyroid dysfunction. The current guidelines of the American Thyroid Association advocate levothyroxine treatment of subclinical hypothyroidism only in TPOAb positive women, while the European Thyroid Association and Endocrine Society guidelines do not incorporate TPOAb status in their recommendations.[1-3]

Thyroid autoimmunity decreases the functional capacity of the thyroid gland, ultimately leading to thyroid failure and hypothyroidism. Already before the onset of hypothyroidism, the decreased capacity may become apparent during a state of increased demand such as early pregnancy. This increased demand is in part mediated by factors that lower thyroid hormone availability such as an increase in thyroxine binding globulin, thyroxine transport to the fetus and thyroid hormone degradation by placental deiodinase type 3. On the other hand, high concentrations of hCG stimulate the thyroid, overall leading to a net increase in thyroid hormone availability during early pregnancy. The decreased thyroid functional capacity due to autoimmunity is supported by observations that TPOAb positive women have higher median TSH concentrations and are at approximately 8-fold higher risk to develop subclinical hypothyroidism during pregnancy.[6,7] Poppe *et al.* (N=35) showed that TPOAb positive women undergoing assisted reproductive therapy have an attenuated FT4 response to synthetic hCG administration and Glinoer et al. demonstrated an attenuation in the classical TSH dip during early pregnancy in TPOAb positive.[6,8] However, the effects of TPOAb positivity on thyroid stimulation by hCG during pregnancy are currently unknown.

It has consistently been shown in different studies, including the Generation R study, that TPOAb positivity is associated with a higher risk of premature delivery.[2-4,9-11] However, the pathophysiological mechanism underlying this association remains to be elucidated. Because TPOAbs increase the risk of thyroid dysfunction, the higher risk of premature delivery in TPOAb positive women may be mediated via alterations in thyroid function. Nevertheless, we previously showed that the association of TPOAb positivity with premature delivery does not change after additional adjustment for TSH and FT4, which might suggest an effect independent of maternal thyroid function.[10] An alternative explanation would be that TPOAb positivity is a reflection of a higher susceptibility to autoimmunity in general and that autoimmune processes confound the association of TPOAb positivity with premature delivery.[12-14]

We hypothesized that TPOAb positive women have an inappropriate thyroidal response to hCG stimulation (as a marker for an abnormal thyroid functional capacity) and that this would be reflected by an attenuated association of hCG with FT4 and TSH during pregnancy in TPOAb positive women. Moreover, we speculated that an attenuated association of hCG with FT4 and TSH may underlie the association of TPOAb positivity with premature delivery.

MATERIALS AND METHODS

Design

We tested these hypotheses in two large prospective cohorts: the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands[15] and in the Holistic Approach to Pregnancy and the first Postpartum Year (HAPPY) study, a population-based prospective pregnancy cohort in the Eindhoven area, the Netherlands.[16]

In Generation R, a total of 7069 women with a delivery date between April 2002 until January 2006 were enrolled during early pregnancy (<18 weeks) in hospitals and midwife practices.[15] Blood samples were drawn in 6398 of these women and for 6278 women enough material was present to measure TSH, FT4, TPOAb or hCG. Women with twin pregnancies (N=128), women that underwent fertility treatment (N=76), women with pre-existing thyroid disease or thyroid (interfering) medication usage (N=89; assess three times during pregnancy by questionnaire), women with missing data on gestational age at birth or a difference of more than one week in gestational age at blood sampling between the hCG and TSH or FT4 samples (N=47) were excluded. For analyses on the association of hCG with thyroid function, we performed a complete-case analysis in 5435 women during early pregnancy (median week 12, 95% range: 11-18) in which enough serum was available to measure TSH, FT4, TPOAbs and hCG. Gestational age at birth was obtained for >99% of all participating mother-child pairs. The Netherlands is generally an iodine sufficient country, as reflected by the iodine status in the Generation R study (median population urinary iodine 225 μg/L).[17]

In the HAPPY study, eligible mothers were those who presented at any of 17 primary care community midwife practices in the area of South-East Brabant, from January 2013 until September 2014.[16] A total of 2130 women were enrolled (<25 weeks; 95% enrolled <18 weeks) and in 1706 women blood samples from early pregnancy were available. In 1563 women, a blood sample from late pregnancy (median week 32, 95% range 31-35) was also available. TSH, FT4 and hCG were measured during early pregnancy in 1706 women with a singleton pregnancy (median week 13, 95% range: 11-18) and during late pregnancy in 1606 women with a singleton pregnancy (median week 33, 95% range: 31-35). Women with pre-existing thyroid disease or thyroid (interfering) medication usage (N=43) were excluded, no data was available on fertility treatment. Further details on data ascertainment are presented in the supplemental appendix.

Clinical outcomes

In order to study whether differences in the association of hCG with thyroid function may explain the association of TPOAb positivity with a higher risk of premature delivery, we used the same study population that we have previously used to study the associations of TPOAb positivity with premature delivery.[10] In short, data on gestational age at birth was obtained from community midwives, obstetricians, and hospital registries. Premature delivery was defined as the onset of premature labor before the 37th of gestation. In addition we also performed a sensitivity analysis on spontaneous premature delivery which was defined as a spontaneous onset of premature labor before the 37th week of gestation and included women who delivered without induction of labor or an elective caesarean section. After exclusion of women with twin pregnancies (N=128), preexisting thyroid disease or thyroid interfering medication usage (N=89) and women with fertility treatment (N=76)[10] and additional exclusion of women with hCG measurement that were not performed within the same week as thyroid function measurement (N=15), data on gestational age at birth and TSH, FT4, TPOAb or hCG concentrations measured in the same sample were available in 5956 women from the Generation R study (missing data on serum TSH, FT4 or hCG (randomly missing due to lack of serum in 6.3%, 5.7%, 3.5%, respectively) were imputed to

allow comparison of the new analyses to previously published analyses).[10] Sensitivity analyses for the risk of premature delivery were performed in nulliparous women only, because nulliparous women are at higher risk for TPOAb positivity,[18] and in women with spontaneous delivery. We were not able to study the association of TPOAb positivity with premature delivery in the HAPPY study, because only 125 women were TPOAb positive (7.3%) and 70 women (4.2%) had a premature delivery. Between-study differences in thyroid function, TPOAb and hCG measurements (serum versus plasma, assay differences) did not allow for meta-analysis.

Statistical analysis

The association of hCG concentrations with FT4 and TSH concentrations were studied using linear regression models. We tested for potential effect modification in the association of hCG with FT4 or TSH by TPOAb positivity by adding the product interaction term for TPOAb positivity (yes/no)*hCG to the model. Gestational age at birth was studied using multivariable linear regression models. Premature delivery was studied using multivariable logistic regression models. We investigated interaction between TPOAb positivity and hCG for gestational age at birth or premature delivery by introducing a product term to the model and subsequent plotting of interactions. In order to investigate if differences in the risk of premature delivery between TPOAb positive and TPOAb negative women differed according to FT4 and hCG, all two-way and the three-way interaction of FT4, hCG and TPOAb positivity (yes/no) were added to the models. In case of a significant three-way interaction, analyses of TPOAb positivity as a risk factor for premature delivery were stratified according to FT4 concentrations standardized to hCG.

In this study, we defined the expected thyroidal response to hCG stimulation cross-sectionally based on the assumption that the predicted means in the whole population are the best approximation of hCG mediated FT4 changes in an individual. FT4 concentrations standardized to hCG were defined by the residuals of a regression model in which hCG was regressed on FT4 in TPOAb negative women. Since the response of FT4 to hCG is most pronounced in women with high concentrations of hCG, women with relatively low hCG concentrations in this cross-sectional study are more prone to misclassification for their expected thyroidal response to hCG. In other words, the FT4 response in women with low hCG may change when higher hCG concentrations are present. Therefore, we also performed the stratified analyses on the risk of premature delivery in women with an hCG above the median only (Generation R median = 45,000 IU/L, depicted by line 'a' in Figure 4). Between-group median TPOAb concentrations were calculated using Mood's median test using logtransformed TPOAb concentrations. Further details about the statistical analyses are reported in the appendix.

All statistical analyses were performed using Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc. Chicago, IL, USA) or using R statistical software version 3.03 (packages *rms*, *hmisc* and *visreg*).

RESULTS

Ìn Generation R, after exclusions a total of 5956 women were enrolled during early pregnancy (median [95% range]: 13.2 weeks [9.6-17.6]; Figure 1). There was no difference in urinary iodine/creatinine ratio between TPOAb negative and positive women (283 *vs.* 291 µg/g; *P*=0.61). In HAPPY, after exclusions a total of 1663 women from were enrolled during early pregnancy (13.0 weeks [11.0-19.0]), and 1563 women during late pregnancy (32.3 weeks [31.3-34.9]; Figure 1). Further details on subject characteristics are shown in Supplemental Table 1.

FIGURE 1. *Flowchart of selection procedure for both study populations.*

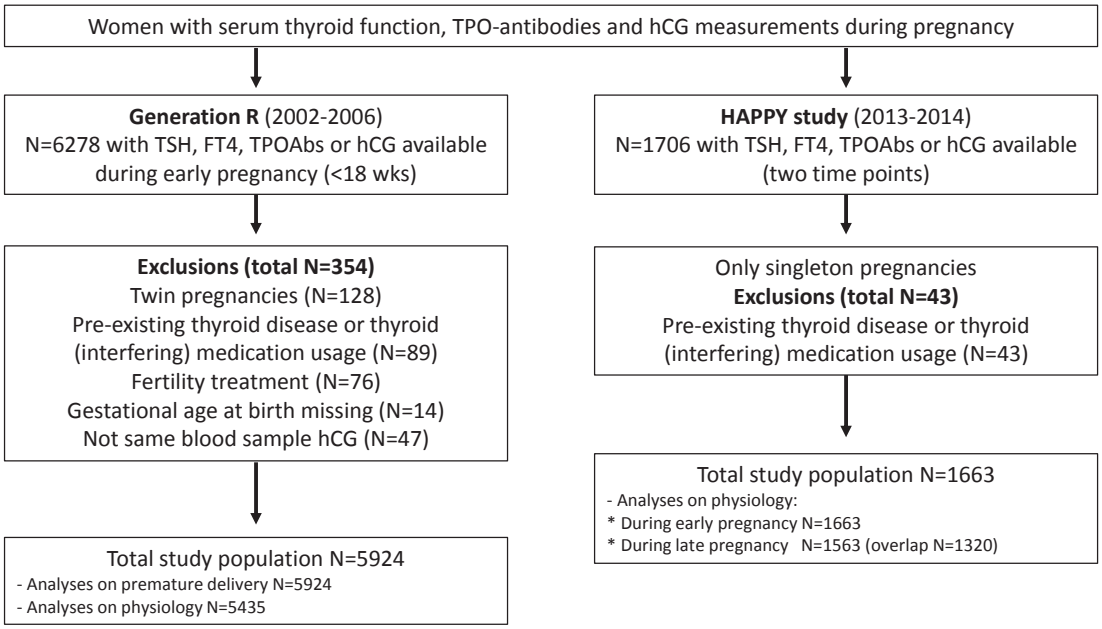
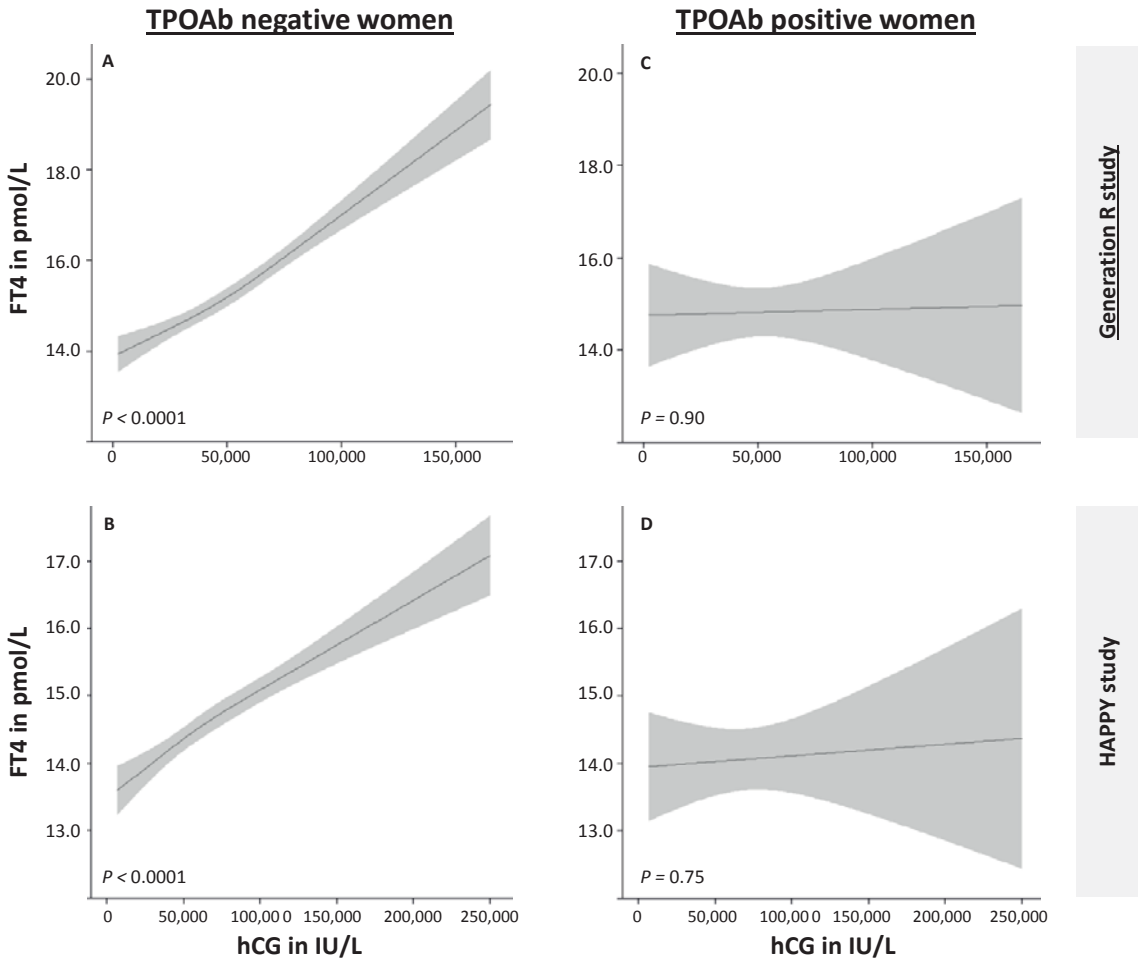


FIGURE 2. *The association of hCG with FT4 stratified according to TPOAb status.*



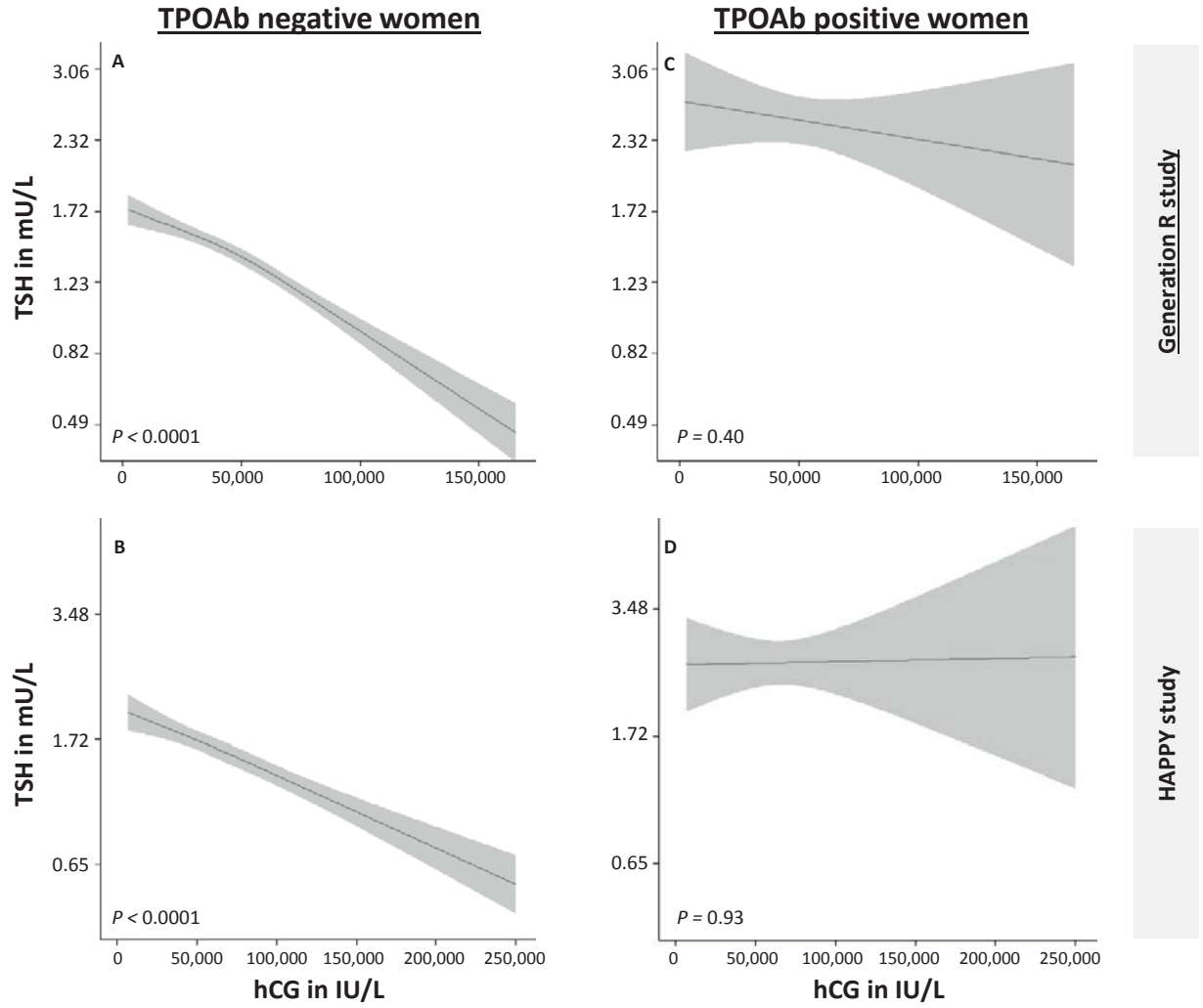
Figures show the association of hCG with TSH in TPOAb negative women (A, B) and TPOAb positive women(C, D) as estimated mean (black line) with 95% confidence interval (grey area).

Association of hCG with thyroid function according to TPOAb status.

In both populations, there was a positive association of hCG with FT4 in TPOAb negative women but not in TPOAb positive women, during early pregnancy (Figure 2; *P* for interaction<0.001 in Generation R and *P* for interaction=0.036 in HAPPY). Through negative feedback, the hCG mediated increase in serum FT4 is subsequently responsible for the decrease in serum TSH concentrations.[5] There was a negative association of hCG with TSH in TPOAb negative women but not in TPOAb positive women, during early pregnancy in both populations (Figure 3; *P* for interaction=0.0077 in Generation R and *P* for interaction=0.0016 in HAPPY).

hCG concentrations were substantially lower in late pregnancy than in early pregnancy (median late pregnancy: 14,000 IU/L versus median 58,000 IU/L during early pregnancy). During late pregnancy, hCG was not associated with FT4 and the negative association of hCG with TSH was attenuated as compared to early pregnancy (Supplemental Figure 1A-B). Longitudinal analyses, comparing the change in hCG concentrations between early and late pregnancy in the HAPPY study, showed that the decrease in hCG was associated with a decrease in FT4 and with an increase in TSH (Supplemental Figure 1C-D).

FIGURE 3. *The association of hCG with TSH stratified according to TPOAb status.*



Figures show the association of hCG with TSH in TPOAb negative women (A, B) and TPOAb positive women(C, D) as estimated mean (black line) with 95% confidence interval (grey area).

TPOAb positivity and the risk of premature delivery.

We subsequently aimed to investigate the potential clinical implications of differences in the thyroidal response to hCG stimulation for the risk of an adverse outcome. Therefore, we investigated if an impaired thyroidal response to hCG may underlie the higher risk of premature delivery in TPOAb positive women by investigating effect modification between hCG and FT4 in logistic regression models for premature delivery.

In TPOAb positive women, differences in hCG modified the association of FT4 with premature delivery (*P* for interaction=0.050) while this was not the case in TPOAb negative women (*P* for interaction=0.33). This effect modification differed between TPOAb positive and TPOAb negative women (Table 1; *P* for three-way interaction=0.012; similar results obtained with gestational age at birth continuously: *P* for three-way interaction=0.045, data not shown).

TABLE 1. *Sensitivity analyses investigating interaction between FT4 and hCG for premature delivery in Generation R (N=5956).*

	Premature delivery	
	TPOAb negative	**TPOAb positive**
Variable in model	*P-value*	*P-value*
FT4	0.90	0.29
hCG	0.17	0.38
Interaction term FT4 * hCG	0.33	0.05
	P for difference: 0.012[a]	

[a] *P-value for three-way interaction (see methods).*

Table 1 shows the P-values for FT4, hCG (separately or combined), and their interaction term from a logistic regression model for premature delivery in TPOAb negative and TPOAb positive women. The results show that in these models, there is significantly different interaction of FT4 and hCG between TPOAb negative and TPOAb positive groups.

TABLE 2. *Stratified analyses of the relationship between TPOAb positivity, hCG and FT4 levels, and the risk of premature delivery.*

	Expected thyroidal response to hCG[a]	**Gestational age (β)**	**Premature delivery (%(N))**	**OR**
TPOAb positivity[b]	Overall	-0.004	7.4% (23/312)	1.66
TPOAb positivity[b]	High (green[b])	-0.002	2.9% (1/35)	0.52
	Above mean (yellow[b])	-0.005	6.3% (6/95)	1.76
	Below mean (orange[b])	-0.006	9.3% (10/107)	2.21
	Low (red[b])	-0.006	8.0% (6/75)	2.27
	In women with hCG above median (>45,000 IU/L)			
	Expected thyroidal response to hCG[a]	**Gestational age (β)**	**Premature delivery (%(N))**	**OR**
TPOAb positivity[b]	Overall	-0.004	6.8% (13/192)	1.60
TPOAb positivity[b]	High (green[b])	+0.008	0.0% (0/17)	*
	Above mean (yellow[b])	-0.001	3.7% (2/54)	0.56
	Below mean (orange[b])	-0.007	7.6% (5/66)	2.33
	Low (red[b])	-0.010	10.7 % (6/56)	2.82

[a] Defined in comparison to mean regression line, decreased: < -1 SD; low normal: -1 – 0 SD; high-normal: 0-1 SD; increased: >1 SD.

[b] As compared to TPOAb negative women.

[c] See Figure 4

* No events; The risk of premature delivery was not different for four groups of hCG mediated FT4 response; P=0.50 amongst all subjects and P=0.87 amongst hCG > 45,000. Beta values for gestational age are transformed by the natural logarithm and estimated using multivariate linear regression models. Analyses on the risk of premature delivery were performed using multivariate logistic regression models. All analyses were adjusted for gestational age at blood sampling, maternal age, smoking, parity, ethnicity and fetal gender.

Figure 4 depicts hCG and FT4 for all women included from Generation R, split into TPOAb negative women (gray dots with grey dotted regression line) and TPOAb positive women (colored dots). TPOAb positive women were grouped according to their expected thyroidal response to hCG as compared to the mean of the TPOAb negative population (expected thyroidal response was defined by the expected mean FT4 based on hCG, ranging from: green - relatively high FT4 for hCG reflecting an above average expected thyroidal response to hCG; to red – relatively low FT4 for hCG reflecting a below average expected thyroidal response to hCG).

The overall risk of premature delivery in TPOAb positive women was 1.7-fold higher than in TPOAb negative women (OR [95%CI]: 1.66 [1.03-2.54]; *P*=0.027). However, when stratified according to the observed expected thyroidal response, TPOAb positive women with an adequate expected thyroidal response to hCG (Figure 4, green group) did not have a higher risk of premature delivery (Table 2). On the other hand, TPOAb positive women with an inadequate expected thyroidal response to hCG (Figure 4, orange and red group) had a 2.2 and 2.3-fold higher risk of premature delivery, respectively (Table 2). Median TPOAb concentrations did not differ between the colored groups (green: 161 IU/L, orange 170 IU/L, yellow: 144 IU/L and red 189 IU/L; *P*=0.46).

FIGURE 4. *hCG and FT4 in TPOAb positive women, relative to expected mean in TPOAb negative women.*

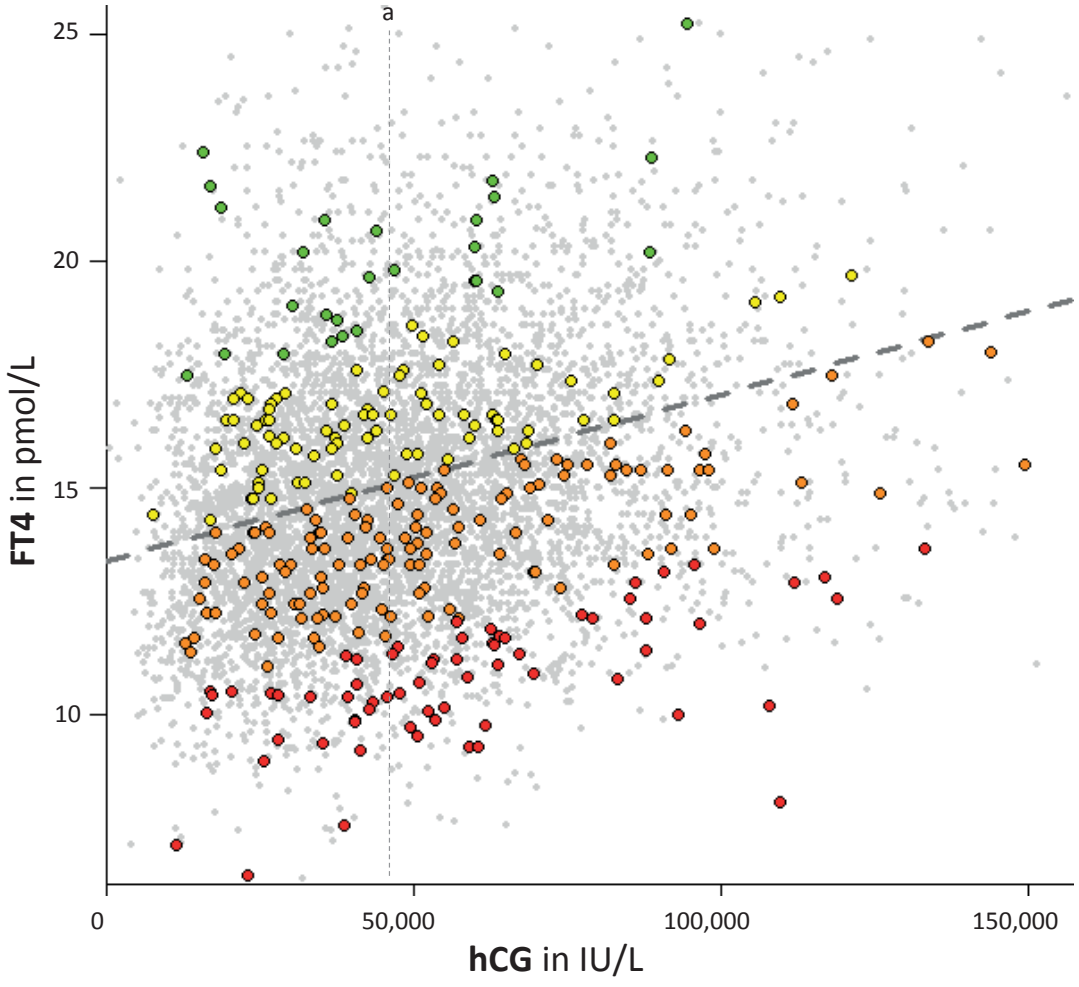


Figure shows the association between hCG and FT4 in the whole population during early as estimated mean (grey line). The colored dots show the TPOAb positive women, the colors mark the distance from the mean expected FT4 level (green: above +1 SD, yellow: 0 tot +1SD, orange: 0 to -1 SD, red: less than -1SD). The vertical dotted line (a) shows the median hCG level used to stratify the analyses (hCG 45,000 IU/L).

TABLE 3. *Stratified analyses of the relationship between TPOAb positivity, hCG and FT4 levels, and the risk of premature delivery.*

		Risk of premature delivery			
	Expected thyroidal response to hCG[a]	**Nulliparous women only (%(N))**	**OR**	**Spontaneous premature delivery (%(N))**	**OR**
TPOAb positivity[b]	Overall	8.9% (17/192)	1.78	7.0% (15/215)	1.77
TPOAb positivity[b]	High (green[b])	4.0% (1/25)	0.45	3.7% (1/27)	*
	Above mean (yellow[b])	5.1% (3/59)	1.18	6.5% (4/62)	1.94
	Below mean (orange[b])	12.3% (8/65)	2.44	9.5% (7/74)	2.41
	Low (red[b])	11.6% (5/43)	4.32	5.9% (3/51)	1.98
	In women with hCG above median (>45,000 IU/L)				
	Expected thyroidal response to hCG[a]	**Nulliparous women only (%(N))**	**OR**	**Spontaneous premature delivery (%(N))**	**OR**
TPOAb positivity[b]	Overall	9.0% (9/100)	1.90	7.6% (9/118)	1.88
TPOAb positivity[b]	High (green[b])	0.0% (0/10)	*	0.0% (0/10)	*
	Above mean (yellow[b])	3.6% (1/28)	0.62	3.2% (1/31)	0.93
	Below mean (orange[b])	12.1% (4/33)	2.89	11.9% (5/42)	2.92
	Low (red[b])	14.3% (4/28)	6.06	8.3% (3/36)	2.63

[a] Defined in comparison to mean regression line, decreased: < -1 SD; low normal: -1 – 0 SD; high-normal: 0-1 SD; increased: >1 SD.
[b] As compared to TPOAb negative women.
[c] See Figure 4
* Not enough events; The risk of premature delivery was not different for four groups of hCG mediated FT4 response; P=0.50 amongst all subjects and P=0.87 amongst hCG > 45,000. Beta values for gestational age are transformed by the natural logarithm and estimated using multivariate linear regression models. Analyses on the risk of premature delivery were performed using multivariate logistic regression models. All analyses were adjusted for gestational age at blood sampling, maternal age, smoking, parity, ethnicity and fetal gender.

A sensitivity analysis in women with relatively high hCG concentrations (above the median, >45,000 IU/L in Generation R) yielded similar results, showing a non-increased risk of premature delivery in TPOAb positive women with an adequate expected thyroidal response to hCG and a 2.3-2.8 fold increased risk of premature delivery in TPOAb positive women with an inadequate expected thyroidal response to hCG (Table 2). Further, sensitivity analyses showed that the differences in the risk of premature delivery were larger in nulliparous women (a risk factor for TPOAb positivity) and similar for the risk of spontaneous premature delivery (Table 3).

DISCUSSION

In this study, we investigated the association of hCG with thyroid function in TPOAb positive and TPOAb negative women during early and late pregnancy using data from two prospective population-based cohorts. The main finding is that in TPOAb positive women hCG is not associated with FT4 or TSH which is in contrast to well-known, strong association of hCG with FT4 and TSH in TPOAb negative women. This difference suggests that TPOAb positivity is associated with an impaired thyroidal response to hCG during pregnancy. Subsequently, we show that TPOAb positive women with an impaired expected thyroidal response to hCG (lower FT4 than expected for hCG) have a higher risk of premature delivery. In contrast, TPOAb positive women with an adequate expected thyroidal response to hCG did not have a higher risk of premature delivery.

Thyroid autoimmunity decreases the functional capacity of the thyroid gland. Outside of pregnancy, early stages of thyroid autoimmunity/TPOAb positivity are marked by a gradual increase in TSH concentrations, which is necessary to keep thyroid hormone availability stable. However, in euthyroid individuals, a decreased thyroid functional capacity may be exposed during a state of increased thyroidal demand such as pregnancy, when high hCG stimulates the thyroid gland to produce more thyroid hormone. As opposed to the increase in TSH concentrations seen in TPOAb positive women outside of pregnancy, TSH receptor stimulation due to hCG during pregnancy has a rapid onset that leads to an increase in thyroid hormone availability and occurs simultaneously with physiological factors that concomitantly decrease thyroid hormone availability.

We show that high hCG concentrations during early pregnancy in TPOAb positive women are not associated with higher FT4 or lower TSH concentrations, which is in contrast to the clear hCG response in TPOAb negative women. These data are in line with a previous study by Poppe *et al.* which showed, in 35 women that received exogenous hCG for assisted reproductive therapy, that TPOAb positive women have an attenuated FT4 response compared to TPOAb negative women.[8] Peak hCG concentrations are found between approximately week 9-12 of pregnancy. Within this critical period of pregnancy, placentation takes place and the fetus depends on maternal THs for its brain development. As a consequence of this timing, an impaired response to hCG stimulation may lead to a relative shortage of THs, particularly during the peak of hCG concentrations. A relative TH shortage during this critical phase in pregnancy may have detrimental effects mimicking those of an absolute TH shortage.[1-4]

Premature delivery has been identified as the largest direct cause of child morbidity and mortality in almost all high and middle-income countries.[19] Furthermore, it is an important risk factor for psychiatric, metabolic, cardiovascular and renal disease later in life.[20-22] The results in this paper provide evidence that the association of TPOAb positivity with a higher risk of premature delivery is mediated by alterations in thyroid function. We demonstrate that there is considerable variation of FT4 response to hCG stimulation within the group of TPOAb positive women. Subsequent analyses suggest that only TPOAb positive women with an inadequate thyroidal response to hCG (FT4 lower than expected

according to hCG) have a higher risk for premature delivery. Interestingly, the benefit of treating TPOAb positive women with levothyroxine for pregnancy outcome is currently studied in two clinical trials [TABLET trial, ISRCTN: 15948785 and T4LIFE trial, NTR3364]. Our findings suggest that particularly those TPOAb positive women that have a decreased expected thyroidal response to hCG may benefit from levothyroxine treatment in these trials.

To our knowledge, this is the first study that investigates the mechanism behind the association of TPOAb positivity and a higher risk of premature delivery. The main strength of this study is the large number of unselected subjects from two independent cohorts with detailed data available on their phenotype, thyroid function, thyroid function determinants and adverse clinical outcome. This enabled us to adjust analyses for various confounding factors and to investigate higher order interactions of continuous variables.

A potential limitation of the current study is that only a single measurement of maternal thyroid function and hCG was available in Generation R. In theory, FT4 concentrations during early pregnancy may have been transient, not reflecting thyroid hormone availability during the full course of pregnancy. However, a single measurement mimics clinical practice during which decisions need to be made as early in pregnancy as possible. Moreover, hCG was only associated with FT4 during early pregnancy and not during the third trimester when hCG concentrations are much lower. This suggests that differences in expected thyroidal response between TPOAb positive and negative women are particularly relevant during early pregnancy when hCG reaches peak concentrations. Another limitation of the cross-sectional design may have been that not all women had hCG concentrations at the time of blood measurement that were high enough to truly distinguish differences in FT4 response between TPOAb positive and negative women. In order to reduce misclassification we performed all analyses in women with hCG above the population median which showed that effect estimates were amplified.

Monitoring the increase in FT4 during pregnancy may be a sensitive manner to identify women with a relative thyroid dysfunction (i.e. relatively low FT4 for hCG), particularly for TPOAb positive women. Such monitoring may be improved through further studies into determinants of the differences in thyroidal response to hCG within the group of TPOAb positive women, for example the co-occurrence of thyroid stimulating antibodies.[23] However, given that hCG peaks early in pregnancy and most women first attend a pregnancy clinic during or after this period, timely initiation of treatment will most likely not be feasible. The development of a test using thyroid stimulation before pregnancy may help to identify TPOAb positive women who are particularly at risk to develop thyroid dysfunction during pregnancy. Such a test would also allow for timely treatment that could even be started before pregnancy.

In conclusion, hCG is not associated with FT4 or TSH in TPOAb positive women, suggesting that TPOAb positivity considerably impairs gestational thyroid stimulation by hCG. Particularly those TPOAb positive women that had a low FT4 relative to their hCG had a higher risk of premature delivery. These results are the first evidence that TPOAb positivity results in adverse pregnancy outcomes via alterations in gestational thyroid function. The abnormal thyroidal response in TPOAb positive women might suggest that these women require a different treatment approach than TPOAb negative women. Future studies are needed to replicate these findings and investigate the potential benefit for screening and subsequent treatment strategies.

SUPPLEMENTARY APPENDIX

Data ascertainment and biochemical measurements

In Generation R, the intra- and interassay coefficients of variation were <4.1% for TSH at a range of 3.97-22.7 mU/L and <5.4% for FT4 at a range of 14.3-25.0 pmol/L (for Vitros ECI; Ortho Clinical Diagnostics, Rochester, NY). Maternal TPOAbs were measured using the Phadia 250 immunoassay (Phadia AB, Uppsala, Sweden) and considered positive when >60 IU/L. Maternal total human chorionic gonadotropin (hCG) concentrations were analyzed in serum using an Immulite XPi system (Siemens Healthcare Diagnostics, Deerfield, IL, USA), details of which have been described previously.[1]

In the HAPPY study, TSH, FT4, TPO-Abs, and hCG are measured in Li-heparin plasma using electrochemoluminescence assays (Cobas® e 601, Roche Diagnostics, Mannheim Germany) and the intra- and interassay coefficients of variation were <2.5% for TSH at a range of 0.05-6.0 mU/L, <3.6% for FT4 at a range of 13-33 pmol/L, 7.5% and 14.6% for TPOAbs at 93 IU/L and 25 IU/L and <3.7% for hCG at a range of 5-200 IU/L. TPOAbs were considered positive at >60IU/L.

In the HAPPY study, early pregnancy TSH and FT4 concentrations did not differ between women with, or women without a second measurement (data not shown). As compared to women with both an early pregnancy measurement and a late pregnancy measurement, women with only an early pregnancy measurement had slightly lower early pregnancy hCG (-0.11 SD; *P*=0.048) and were more likely to be TPOAb positive (34/375 (9.1%) *versus* 77/1295 (5.9%), *P*=0.034). Further details in data ascertainment for Generation R and the Happy study have been described previously.[2, 3]

Statistical analyses (2)

To fulfill model assumptions, TSH and gestational age at birth were logarithmically transformed. Non-linearity was assessed using ordinary least squares linear regression methods utilizing restricted cubic splines with 3 knots at the 10th, 50th and 90th percentile. For all analyses, model fit and remaining model assumptions were assessed by plotting model residuals, evaluating (adjusted) R-squared and/or the le Cessie - van Houwelingen - Copas - Hosmer unweighted sum of squares test. Covariates were added to the models based on biological plausibility, change in the effect estimates of interest and changes in the residual variability of the outcome.

Multiple imputation according to the Markov Chain Monte Carlo method was used for missing data on covariates, five imputed data sets were created and pooled for analyses.[4] In the Generation R database (used to study the association of hCG with FT4 and TSH), maternal smoking, education level, ethnicity, parity and BMI were imputed (missing due to non-response in 13.1%, 7.2%, 4.1%, and <1.0%, respectively). In the HAPPY study database, maternal late pregnancy BMI, late pregnancy gestational age at blood sampling, early pregnancy BMI and early pregnancy gestational age at blood sampling were imputed (missing due to non-response in 10.3%, 4.2% and <1.0%, respectively). No significant differences in descriptive characteristics were found between the original and imputed datasets.

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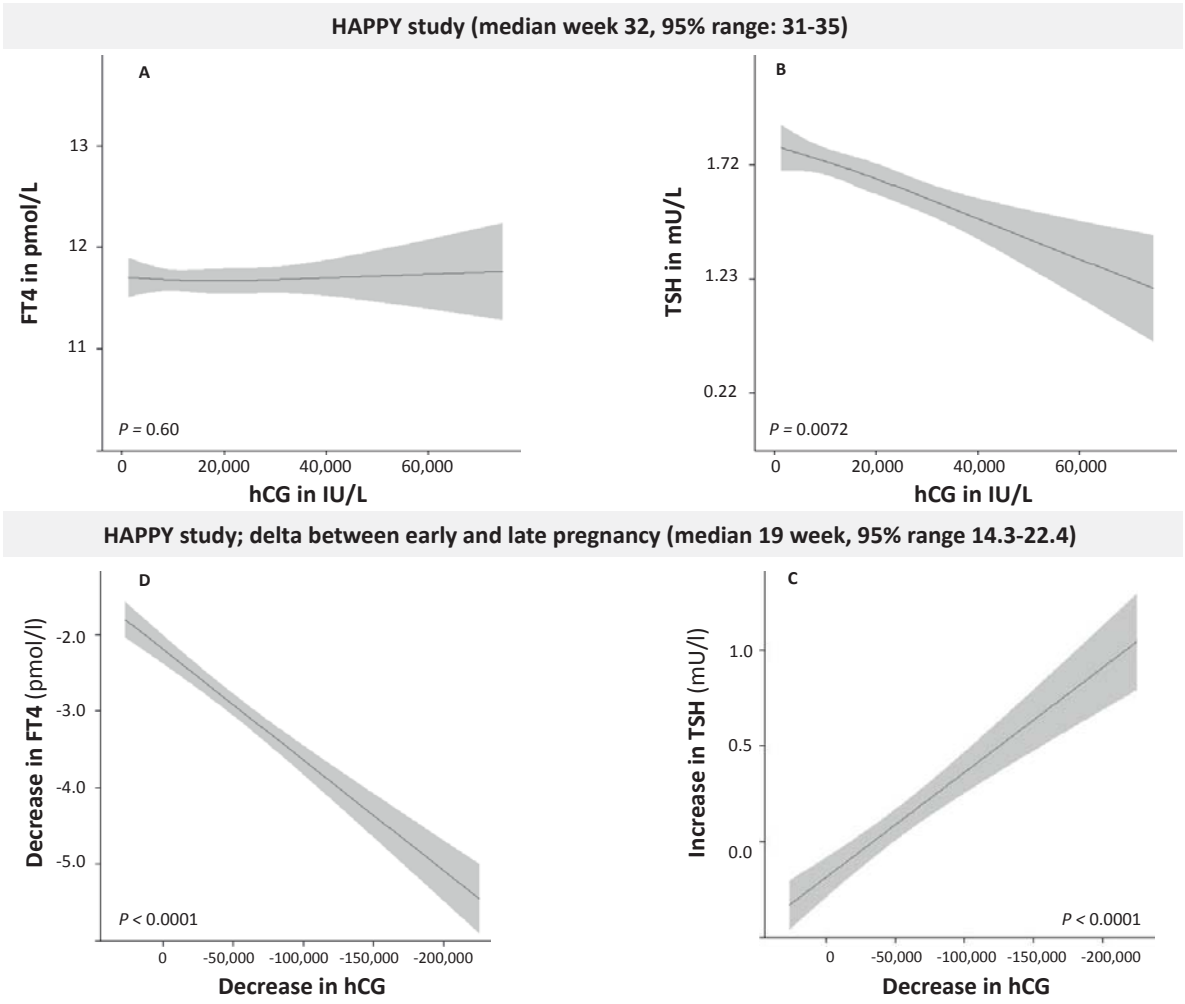
SUPPLEMENTAL TABLE 1. *Characteristics of 5435 women from the Generation R study and 1663 women from the HAPPY study*

		Generation R			HAPPY study	
		Median	**(95% range)**		**Median**	**(95% range)**
			Early pregnancy			
Median TSH	(mU/L)	1.35	(0.04-4.55)		1.46	(0.23-4.60)
Median FT4	(pmol/L)	14.8	(10.3-22.3)		14.4	(11.4-18.0)
Median hCG	(IU/L)	44,416	(11,989-107,273)		58,000	(18,600-175,000)
Gestational age[a]		13.2	(9.6-17.6)		13.0	(11.0-19.0)
TPOAb positivity[f]		5.3%			7.3%	
			Late pregnancy			
Median TSH	(mU/L)	n/a			1.70	(0.54-4.09)
Median FT4	(pmol/L)	n/a			11.7	(9.1-14.7)
Median hCG	(IU/L)	n/a			14,000	(2,400-51,000)
Gestational age[a]		n/a			32.3	(31.3-34.9)
TPOAb positivity[f]		n/a			3.6%	
Maternal age[d]		30.3	(19.5-38.8)		30.0	(23.0-38.0)
BMI early pregnancy		23.6	(18.5-35.8)[e]		23.1	(18.3-33.7)
BMI late pregnancy		n/a			27.0	(21.3-37.3)
Parity[c]						
0		3095	(56.9)[e]		783	(47.1)
1		1633	(30.0)[e]		681	(41.0)
2		508	(9.3)[e]		171	(10.3)
>2		199	(3.7)[e]		28	(1.7)
Smoking[c]						
Non-smokers		3997	(73.5)[e]		1551	(93.3)
Stopped smokers		483	(8.9)[e]		n/a	
Smokers		955	(17.6)[e]		112	(6.7)
Education level						
Low		568	(10.5)[e]		81	(4.8)
Middle		2510	(46.1)[e]		527	(31.6)
High		2357	(43.4)[e]		1055	(63.5)
Ethnicity[c]						
Dutch		2803	(51.6)[e]	Dutch	1620	(97.4)
Moroccan		341	(6.3)[e]	Non-Dutch	43	(2.6)
Turkish		455	(8.4)[e]			
Surinamese		479	(8.8)[e]			
Other western		491	(9.0)[e]			
Other non-western		866	(15.9)[e]			
Child gender[c] (boys %)		2752	(50.6)		823	(49.5)

[a] *At time of blood sampling; Data shown as median in weeks*
[b] *Data shown as mean in grams (SD)*
[c] *Data shown as n (%)*
[d] *Data shown as median in years*
[e] *Data shown after imputation of missing data (13.1% for smoking, 7.2% for education level, 4.1% for ethnicity and <1.0% for BMI and parity).*
[f] *Non-imputed data.*

SUPPLEMENTAL FIGURE 1. *Association of hCG with TSH and FT4 levels during late pregnancy and longitudinally.*



Figures show the association of hCG with either TSH (A, B) or FT4 (C, D) as estimated mean (black line) with 95% confidence interval (grey area).

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CHAPTER 16

THE RISK OF PRE-ECLAMPSIA ACCORDING TO HIGH THYROID FUNCTION IN PREGNANCY DIFFERS BY HCG CONCENTRATION

Tim I.M. Korevaar, Eric A.P. Steegers, Layal Chaker, Marco Medici, Vincent W.V. Jaddoe, Theo J. Visser, Yolanda B. de Rijke and Robin P. Peeters

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ABSTRACT

CONTEXT During pregnancy, there is an increased demand for thyroid hormone. The pregnancy hormone human chorionic gonadotropin (hCG) is an important physiological stimulator of thyroid function. Already high-normal maternal FT4 concentrations are associated with a higher risk of pre-eclampsia.

OBJECTIVE To study our hypothesis that hCG concentrations can distinguish a physiological form of high thyroid function from a more pathological form of high thyroid function, and that the risk of pre-eclampsia would differ accordingly.

DESIGN TSH, FT4, hCG or TPO-antibody levels were determined in pregnant women participating in a population-based prospective cohort study.

SETTING General community.
PARTICIPANTS A non-selected sample of 5146 pregnant women.
INTERVENTIONS None.
MAIN OUTCOME MEASURE(S) Pre-eclampsia.

RESULTS Women with high hCG associated high thyroid function did not have a higher risk of pre-eclampsia than women with normal thyroid function. In contrast, women with low hCG and high thyroid function had a 3.4 to 11.1-fold higher risk of pre-eclampsia. These risk estimates were amplified in women with high BMI. Women with a low hCG and suppressed TSH (<0.10 mU/L) had a 3.2 to 8.9-fold higher risk of pre-eclampsia. hCG was not associated with pre-eclampsia, results remained similar after exclusion of TPO-antibody positive women.

CONCLUSION This study suggests that, in contrast to women with a high hCG associated high thyroid function, women with low hCG and high thyroid function during pregnancy are at higher risk of developing pre-eclampsia. The additional measurement of hCG may therefore help to distinguish a more pathological form of high thyroid function and women at high risk of pre-eclampsia.

INTRODUCTION

Pre-eclampsia affects 2–8% of pregnancies worldwide and is a leading cause of maternal and child morbidity and mortality.[1] Although the prevalence is similar across the globe, large differences between high and low income countries are found for complications and maternal death.[1] Pre-eclampsia is characterized by new onset hypertension and proteinuria after the 20th week of pregnancy and affects multiple organ systems.[1] The pathophysiological mechanisms leading to pre-eclampsia include impaired placentation, trophoblast invasion and uterine spiral artery remodeling, followed by an adverse inflammatory, metabolic and thrombotic response. However, the exact underlying mechanisms remain unknown.

Thyroid hormone plays a role in placental development and is an important regulator of various metabolic and inflammatory processes.[2-6] Overt gestational hyperthyroidism is a known risk factor for pre-eclampsia.[7-10] In line with this, we previously showed that already high-normal levels of FT4 are associated with a 2.1-fold higher risk of pre-eclampsia.[11] However, other studies on subclinical changes in thyroid function have shown conflicting results.[9,10,12-15] This might be due to the underlying mechanism causing high gestational FT4.

High gestational thyroid function is most often caused by a physiological increase in the pregnancy-specific hormone human chorionic gonadotropin (hCG) during early pregnancy.[16] hCG stimulates the thyroid via its affinity with the TSH receptor.[17] hCG concentrations rapidly rise after conception and peak at the end of the first trimester, and consequently, a similar trajectory is seen for FT4 and TSH changes.[16] High gestational thyroid function is typically reported in 40-60% women with hyperemesis gravidarum, a condition that is characterized by high concentrations of hCG.[16,18-21] Nevertheless, hyperemesis gravidarum is associated with favorable pregnancy and child outcomes which suggests that high gestational thyroid function due to high hCG is not pathological.[22]

Alternatively, high gestational thyroid function can be caused by underlying thyroid pathology leading to thyroid hormone overproduction, such as TSH receptor stimulating antibodies, toxic adenomas or goiter. Untreated hyperthyroidism due to these underlying causes is associated with a high risk of adverse outcomes including pre-eclampsia.[10,23]

We hypothesized that additional measurement of hCG at the time of thyroid function assessment can distinguish physiological from non-physiological changes in thyroid function during pregnancy. In the current study we therefore investigate if women with high hCG associated high thyroid function (high hCG with high FT4 or low TSH) have a different risk of pre-eclampsia than women with low hCG and high thyroid function (low hCG with high FT4 or low TSH).

MATERIALS AND METHODS

In order to investigate this hypothesis, TSH, FT4, TPO-antibodies (TPOAbs) and hCG levels were determined in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[24] We have previously described the association of TSH and FT4 during early pregnancy with the development of pre-eclampsia.[25] We now additionally measured hCG concentrations which enables us to investigated whether the addition of a hCG measurement would improve the interpretation of maternal thyroid function in the risk assessment of pre-eclampsia using the same database. Population for analyses, covariates and definitions of outcomes have been described previously.[25] In short, data on early pregnancy TSH or FT4 (<18 weeks' gestation) and pre-eclampsia was available for 5803 women. Women with twin pregnancies (N=128), preexisting thyroid

disease or thyroid interfering medication usage (N=85) and women with fertility treatment (N=68) were excluded and if subsequent pregnancies were recorded in the database, only the data from the first recorded pregnancy was used (N=369 excluded). Additionally, women were excluded in case the hCG measurement could not be performed within the same sample as thyroid function was measured (N=7). Certified medical doctors reviewed women's hospital charts and defined pre-eclampsia according to the criteria of the International Society for the Study of Hypertension in Pregnancy.[26,27] Pre-eclampsia was identified as the development of a systolic BP of 140 mm Hg or greater and/or a diastolic BP of 90 mm Hg or greater (at least two BP readings) after 20 weeks of gestation in a previously normotensive woman, plus the presence of proteinuria (defined as two or more dipstick readings of 2 or greater, one catheter sample reading of 1or greater, or a 24 h urine collection containing at least 300 mg of protein). All analyses were adjusted for gestational age at blood sampling, maternal age, BMI, smoking, parity, education level, ethnicity, and fetal gender.

Statistical analysis

We used logistic regression models utilizing restricted cubic splines with 3 knots to assess non-linearity of the associations between TSH, FT4, hCG and pre-eclampsia. Removal of outliers for TSH, FT4 or (gestational age adjusted) hCG did not change the results. We investigated if the association of FT4 with pre-eclampsia differed according to hCG by adding a product interaction term to the model after selecting TPOAb negative women without high hCG (arbitrarily predefined as below the population median or <45.000 IU/L; in order to overcome misclassification). For the interaction term, a *P*-value of <0.15 was considered for subsequent stratification.[28] To investigate consistency of the association analyses were stratified according to tertiles (main analysis) as well as quartiles of hCG. Given the strong association of FT4 with TSH, we also stratified the effects of TSH for hCG even though we previously did not find an association of TSH with pre-eclampsia. TSH was assessed according to quintiles, and in addition we also used TSH concentrations that reflect clinical TSH suppression (<0.4 and <0.1 mU/L) because the effects of TSH in our previous study displayed a less strong association. For variables with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used.[29] Five imputed data sets were created and pooled for analyses. TSH, FT4, hCG, smoking, education level, ethnicity, parity, BMI and gestational age at blood sampling were added to the imputation model (missing due to non-response/non-recording in 1.5%, 0.6%, 3.7%, 12.4%, 7.4%, 5.9% and all other variables <2.0%, respectively). Furthermore, we added maternal age, fetal gender and TPOAb levels as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. All statistical analyses were performed using statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA).

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RESULTS

After exclusions, the final study population comprised 5146 women. Descriptive characteristics of the study population are shown in Table 1. Pre-eclampsia occurred in 2.6% of all women. The study population comprised mainly nulliparous (60.9%), Dutch (50.9%), non-smoking women (72.6%) and was iodine sufficient (median urinary iodine/creatinine ratio 274 µg/g).

The association of high thyroid function with pre-eclampsia

The association of FT4 with pre-eclampsia differed between women with low and high hCG (product interaction term of continuous variables *P*interaction=0.036) and we subsequently stratified analyses.

hCG concentrations were not associated with the risk of pre-eclampsia (hCG concentrations continuously per 10.000 IU/L: β 1.03 (0.96-1.11), *P*=0.40); Compared to middle tertile, OR PE lowest tertile: 0.93 (0.60-1.43), *P*=0.73; OR PE highest tertile: 1.09 (0.71-1.67), *P*=0.69). When stratified according to hCG, women with low hCG and high FT4 had a 4.6-fold higher risk of pre-eclampsia (Table 2), whereas women with a high hCG associated high FT4 did not have an higher risk of pre-eclampsia (Table 2). Similar results were obtained with a different cut-off for high FT4, after exclusion of TPOAb positive women (Table 2) and according to different cut-offs for hCG (Supplemental Table 1).

TABLE 1. *Descriptive statistics of 5146 women from the Generation R study.*

		Median	(95% range)
TSH	(mU/L)	1.35	(0.04 - 4.45)
FT4	(pmol/L)	14.8	(10.3 - 22.4)
hCG	(IU/L)	44,285	(12,030 – 107,312)
Iodine to creatinine ratio*	(µg/g)	274	(93–770)
TPOAb positivity (%)		5.5%	
Pre-eclampsia (%)		2.6%	
Gestational age		13.2	(9.8 - 17.5)**
Maternal age		30.1	(19.4 – 38.9)
BMI		23.6	(18.5 - 35.8)
Parity			
0		3132	(60.9)
1		1341	(26.1)
≥2		673	(13.1)
Smoking			
Non-smokers		3740	(72.6)
Stopped smokers		486	(9.5)
Smokers		920	(17.9)
Education level			
Low		558	(10.8)
Middle		2415	(46.9)
High		2174	(42.3)
Ethnicity			
Dutch		2624	(50.9)
Moroccan		323	(6.3)
Turkish		450	(8.7)
Surinamese		623	(12.1)
Other western		619	(12.0)
Other non-western		510	(9.9)
Child gender[c] (boys %)		2614	(50.8)

** In random subset of urine samples from N=946 women*
*** Full range = 6-18 weeks.*
Data shown after imputation of missing data (see methods).

TABLE 2. *The association between high FT4 and the risk of preeclampsia stratified for hCG.*

	hCG level	Preeclampsia (%(N))		OR (95%CI)		TPOAb+ excluded OR (95%CI)	
Highest FT4 quintile	Any	3.1%	(31/985)	1.72	(0.96-3.07)	1.90	(1.02-3.52)
	< 35,000	5.3%	(11/208)	4.64	(1.44-15.0)	4.88	(1.50-15.9)
	35,000 – 55,000	1.7%	(5/336)	0.61	(0.19-1.92)	0.62	(0.17-2.28)
	> 55,000	3.1%	(15/487)	1.80	(0.67-4.83)	1.74	(0.58-5.21)
FT4 ≥ 90th percentile[b]	Any	3.5%	(18/519)	1.50	(0.89-2.52)	1.59	(0.93-2.72)
	< 35,000	5.9%	(10/170)	3.44	(1.61-7.39)	3.97	(1.12-8.68)
	35.,000 – 55,000	1.2%	(2/168)	0.39	(0.09-1.74)	0.19	(0.02-1.58)
	> 55,000	3.3%	(6/181)	1.43	(0.58-3.55)	1.73	(0.68-4.44)

[a] As compared to the 3rd quintile [b] As compared to the rest of the population
Any analyses were adjusted for gestational age at blood sampling, maternal age, smoking, education level, ethnicity, parity, BMI and fetal gender. Additional adjustment for hCG and/or exclusion of abnormal gestational age specific hCG values (highest and lowest 1% MoM hCG value) did not change the results.

When stratified according to hCG, women with high hCG associated fully suppressed TSH (high hCG and TSH <0.10 mU/L) did not have a higher risk of pre-eclampsia while women with low hCG and fully suppressed TSH (relatively low hCG and TSH <0.10 mU/L) had a 4.3-fold higher risk of pre-eclampsia (Table 3). Women with low TSH (lowest quintile), or women with suppressed TSH (<0.4 mU/L) did not have a higher risk of pre-eclampsia when stratified for hCG (Table 3). Further stratification using different hCG cut-offs suggested that suppressed TSH in the presence of very low levels of hCG was associated with a higher risk of pre-eclampsia, but this occurred in a very small number of women (Supplemental Table 2).

TABLE 3. *The association between low TSH and the risk of preeclampsia stratified for hCG.*

	hCG level	Preeclampsia (%(N))		OR (95%CI)		TPOAb+ excluded OR (95%CI)	
Fully suppressed <0.1 mU/L	Any	5.1%	(9/178)	2.27	(1.11-4.67)	1.97	(0.88-4.43)
	< 35,000	9.4%	(3/32)	4.25	(1.17-15.5)	3.16	(0.68-14.7)
	35,000 – 55,000	2.9%	(1/34)	1.02	(0.12-8.43)	1.07	(0.13-8.96)
	> 55,000	4.4%	(5/113)	2.08	(0.74-5.80)	1.52	(0.48-4.83)
Suppressed <0.4 mU/L	Any	2.8%	(12/423)	1.26	(0.68-2.33)	1.13	(0.58-2.22)
	< 35,000	3.8%	(3/378)	1.71	(0.50-5.87)	1.17	(0.26-5.18)
	35,000 – 55,000	2.1%	(2/96)	0.76	(0.18-3.32)	0.78	(0.18-3.46)
	> 55,000	2.8%	(7/246)	1.32	(0.55-3.13)	1.01	(0.39-2.63)
1st TSH quintile[a]	Any	3.2%	(33/1024)	1.45	(0.85-2.46)	1.30	(0.74-2.29)
	<35,000	3.1%	(7/224)	1.09	(0.41-2.90)	0.81	(0.27-2.42)
	35,000–55,000	1.9%	(6/308)	1.97	(0.54-7.20)	1.98	(0.54-7.28)
	> 55,000	4.0%	(20/495)	1.27	(0.56-2.88)	1.11	(0.44-2.83)

[a] As compared to the 3rd quintile
** Too smAny groups for reliable analyses*
Any analyses were adjusted for gestational age at blood sampling, maternal age, smoking, education level, ethnicity, parity, BMI and fetal gender. Additional adjustment for hCG and/or exclusion of abnormal gestational age specific hCG values (highest and lowest 1% MoM hCG value) did not change the results.

The association of high thyroid function among women with pre-eclampsia risk factors.
High BMI and nulliparity are known risk factors for pre-eclampsia. Among women with a high BMI (>25 kg/m^2), women with high FT4 (highest quintile) had a 2.9-fold higher risk of pre-eclampsia (Table 4). Stratification for hCG showed that women with low hCG and high FT4 had a 7.7 to 8.3-fold higher risk of developing pre-eclampsia (Table 4; the number of women with TSH <0.1mU/L was too low to perform reliable analyses).

Among nulliparous women, the risk of pre-eclampsia was 1.6-fold higher in women with high-normal FT4, but this analysis did not reach statistical significance (Table 4). Similar to the analyses among women with any parity, stratification for hCG showed that only women with low hCG and high FT4 were at higher risk of developing pre-eclampsia (Table 4). Among women with a high BMI or nulliparous women, low-normal or suppressed TSH was not associated with the risk of pre-eclampsia, also not after stratification for hCG (Table 4).

All analyses remained similar after additional adjustment for hCG concentrations within each hCG strata and after exclusion of women with high or low (<1st or >99th percentile) gestational age specific hCG.

TABLE 4. *The association between high thyroid function and the risk of preeclampsia stratified for hCG stratified by known risk factors for preeclampsia.*

	hCG level	BMI > 25 OR (95%CI)	Only nulliparous OR (95%CI)
	Analyses on FT4		
Highest FT4 quintile	Any	2.91 (1.26-6.74)	1.60 (0.85-3.04)
	< 35,000	7.71 (1.93-30.8)	5.20 (1.31-20.6)
	35,000 – 55,000	1.26 (0.18-8.90)	0.67 (0.20-2.27)
	> 55,000	0.98 (0.21-4.68)	1.65 (0.55-4.95)
FT4 ≥ 90th percentile	Any	3.04 (1.55-5.96)	1.61 (0.91-2.87)
	< 35,000	8.28 (3.30-20.8)	3.95 (1.64-9.52)
	35,000 – 55,000	0.35 (0.04-3.37)	0.47 (0.10-2.18)
	> 55,000	1.36 (0.24-7.73)	1.44 (0.53-3.88)
	Analyses on TSH		
1st TSH quintile[a]	Any	1.22 (0.56-2.65)	1.36 (0.75-2.47)
	< 35,000	1.68 (0.48-5.96)	0.92 (0.26-3.20)
	35,000 – 55,000	2.56 (0.42-15.8)	2.00 (0.52-7.78)
	> 55,000	0.68 (0.17-2.63)	1.19 (0.49-2.87)
Suppressed <0.4 mU/L	Any	1.63 (0.70-3.76)	1.12 (0.55-2.31)
	< 35,000	3.52 (0.88-14.1)	0.75 (0.09-6.02)
	35,000 – 55,000	1.50 (0.29-7.72)	0.95 (0.21-4.29)
	> 55,000	1.02 (0.18-5.69)	1.34 (0.52-3.44)

[a] *As compared to the 3rd quintile*
Any analyses were adjusted for gestational age at blood sampling, maternal age, smoking, education level, ethnicity, parity, BMI and fetal gender. Additional adjustment for hCG and/or exclusion of abnormal gestational age specific hCG values (highest and lowest 1% MoM hCG value) did not change the results.

DISCUSSION

In the current study, we demonstrate that women with a high thyroid function have a different risk of developing pre-eclampsia depending on the hCG concentration. The main finding is that women with a high hCG associated high FT4 do not have a higher risk to develop pre-eclampsia. In contrast, women with a low hCG and high FT4 have a 3.4 to 4.9-fold higher risk to develop pre-eclampsia. We also show that the association of high FT4 with pre-eclampsia is amplified in women with a high BMI, but similar in nulliparous women.

The results of this study support the concept that thyroid function measurements that are incoherent with concomitantly measured hCG concentrations may represent an underlying pathological process (including TSH receptor stimulating antibodies, toxic adenoma, toxic goiter) and support the general notion that high thyroid function due to high hCG should not be treated with antithyroid drugs. In contrast, women with low hCG and high thyroid function may be considered as a high risk group.

We previously showed that the overall association of FT4 with pre-eclampsia is stronger than the associations for TSH.[11] Although the effects of FT4 were in line with the effect of TSH in the current study, our data suggest that the high risk group is best defined based on the measurement of hCG and FT4. This might be explained by the physiological process involved, in which hCG directly increases FT4 production via stimulation of the thyroid whereas TSH suppression is subsequently effectuated via the negative feedback loop. Further studies are needed to confirm the identification of high risk groups and to assess to what extent those women could benefit from a more thorough diagnostic work-up, more regular follow-up visits and/or treatment with low dose antithyroid drugs.

Previous studies have shown that untreated gestational hyperthyroidism due to underlying thyroid pathology is associated with severe outcomes and a high risk of developing pre-eclampsia.[10,23] Underlying thyroid pathologies can be difficult to distinguish during pregnancy from transient thyrotoxicosis due to high hCG concentrations. The additional measurement of hCG can be used to identify women that are likely to have a high thyroid function due to underlying thyroid pathology, i.e. women presumed to have a more pathological cause of high thyroid function (low hCG with high FT4 or low TSH). Alternatively, high gestational thyroid function despite low hCG may be due to genetic variance.[30]

This is the first study that suggests that subclinical forms of hyperthyroidism pathology, as identified by an additional hCG measurement, may increase the risk of pre-eclampsia. Most likely, this higher risk is mediated via synergistic effects of autonomous thyroid hormone production or TSH receptor antibodies and hCG stimulation that lead to a more sustained and pronounced increase in thyroid hormone production. Interestingly, in conditions such as preeclampsia higher relative amounts of more potent hCG isoforms, such as hyperglycosylated or nicked hCG, have been shown.[31,32] Therefore, an alternative explanation for our findings is therefore that the combination of a subclinical form of hyperthyroidism pathology together with a relatively larger concentration of hCG isoforms with a higher TSH receptor stimulating potential synergistically cause the incoherent combination of thyroid function tests with total hCG concentrations.[3] Further studies are needed to identify mechanisms that lead to the biochemical phenotype of low hCG and high gestational thyroid function.

High BMI and nulliparity are well-known risk factors for pre-eclampsia.[1] Reflective of the multifactorial pathophysiology of pre-eclampsia, we show that women with both a low hCG and high thyroid function, as well as a high BMI have a very high risk of pre-eclampsia. However, a higher BMI is associated with a lower gestational thyroid function [34-36]. Therefore, it can also be argued that the underlying cause of high thyroid function is more severe in women with a high BMI, as in these women a slightly higher thyroid function would require more thyroid hormone production. Interestingly, a high BMI is also associated with lower hCG.[37] Therefore, an alternative explanation for our results is that women with a high BMI

are more likely to be adequately categorized as having a low hCG and high thyroid function. In contrast to a high BMI, nulliparity is associated with higher hCG and no clear association with thyroid function has been described.[37-39] This may explain why we did not find any amplification of the effect of the combination of low hCG and high thyroid function on the risk of pre-eclampsia in nulliparous women.

Because women with low hCG and high thyroid function can only be distinguished from high hCG associated high thyroid function when hCG levels are low. As a consequence, it seems likely that the biochemical combination of low hCG and high thyroid function can only be distinguished outside of the peak in hCG, which occurs anywhere between the 9th and 12th week of pregnancy. In this study a low hCG (defined as a hCG <35,000 IU/L) occurred in 33.3% of all participants with a median and 95% range for gestational age of blood sampling of 15.1 (11.4-17.8) weeks. As such, repeated thyroid function testing or adequate timing of blood sampling are required to identify women with an abnormal combination of hCG and TSH or FT4.

To our knowledge, this is the first study that aims to utilizes hCG to distinguish a physiological from a more pathological form of high thyroid function and subsequently study the effects on a clinical outcome. We were able to study this in a large population of unselected pregnant women with detailed phenotype data. A potential limitation of this study is the relatively small number of women with pre-eclampsia. Because the combination of low hCG with high thyroid function also did not occur often, our final analyses compared relatively small groups and odds ratios may be inflated, especially for analyses in women with a hCG <20,000 IU/L (Supplemental Table 1 & 2). However, we found that the association of low hCG and high thyroid function with pre-eclampsia was consistent and dose-dependent, and also that the addition of a product interaction term of continuous variables into the model was highly suggestive of effect modification. Another potential limitation of our study is that only one measurement of maternal thyroid function was available and therefore a high FT4 or low TSH in this study could have been transient. However, the identification of women with autonomous thyroid hormone production will not be affected by this as these women are more likely to have a less transient course, or more persistent form of high thyroid function throughout pregnancy. Unfortunately, we did not have further data available that would further differentiate aetiologies of high thyroid function, further evaluation of potential aetiologies is proposed for future studies.

In conclusion, in contrast to women with high hCG associated high thyroid function, women with low hCG and high thyroid function during pregnancy are at higher risk of developing pre-eclampsia. The additional measurement of hCG may help to distinguish a physiological from a more pathological cause of high thyroid function. Further studies are needed to investigate the role of different hCG isoforms and to further explore optimal timeframes during which hCG measurement add to the interpretation of thyroid function.

APPENDIX

SUPPLEMENTAL TABLE 1. *The association between high FT4 and the risk of preeclampsia stratified for hCG.*

	hCG level	Preeclampsia (%(N))		OR (95%CI)		TPOAb+ excluded OR (95%CI)	
Highest FT4 quintile	Any	3.1%	(31/985)	1.72	(0.96-3.07)	1.90	(1.02-3.52)
	< 20,000	7.5%	(4/53)	11.1	(1.12-110)	12.8	(1.20-137)
	20,000 – 40,000	3.5%	(8/226)	2.92	(0.89-9.64)	3.22	(0.87-12.0)
	40,000 – 60,000	1.4%	(4/283)	0.62	(0.18-2.17)	0.68	(0.19-2.50)
	> 60,000	3.6%	(15/423)	1.90	(0.66-5.46)	2.17	(0.67-7.02)
FT4 ≥ 90th percentile	Any	3.5%	(18/519)	1.50	(0.89-2.52)	1.59	(0.93-2.72)
	< 20,000	7.5%	(4/53)	9.03	(2.16-37.5)	11.1	(2.36-52.0)
	20,000 – 40,000	4.3%	(7/164)	2.13	(0.90-5.07)	2.12	(0.84-5.36)
	40,000 – 60,000	0.6%	(1/162)	0.25	(0.03-1.92)	0.23	(0.03-1.82)
	> 60,000	4.3%	(6/140)	1.80	(0.71-4.58)	2.21	(0.85-5.75)

[a] As compared to the 3rd quintile
Any analyses were adjusted for hCG, gestational age at blood sampling, maternal age, smoking, education level, ethnicity, parity, BMI and fetal gender.
Additional adjustment for hCG and/or exclusion of abnormal gestational age specific hCG values (highest and lowest 1% MoM hCG value) did not change the results.

SUPPLEMENTAL TABLE 2. *The association between low TSH and the risk of preeclampsia stratified for hCG.*

	hCG level	Preeclampsia (%(N))		OR		TPOAb+ excluded OR (95%CI)	
1st TSH quintile[a]	Any	3.2%	(33/1024)	1.45	(0.85-2.46)	1.30	(0.74-2.29)
	< 20,000	2.8%	(2/72)	0.73	(0.12-4.50)	0.44	(0.04-4.83)
	20,000 – 40,000	2.7%	(6/226)	1.49	(0.49-4.48)	1.20	(0.37-3.87)
	40,000 – 60,000	2.3%	(7/310)	1.15	(0.37-3.64)	1.38	(0.41-4.65)
	> 60,000	4.3%	(18/416)	1.51	(0.60-3.80)	1.38	(0.20-2.81)
Suppressed <0.4 mU/L	Any	2.8%	(12/423)	1.26	(0.68-2.33)	1.13	(0.58-2.22)
	< 20,000	5.9%	(1/17)	3.56	(0.35-36.3)	*	
	20,000 – 40,000	3.4%	(3/87)	1.48	(0.43-5.07)	1.59	(0.46-5.52)
	40,000 – 60,000	2.0%	(2/102)	0.89	(0.21-3.84)	0.89	(0.20-3.89)
	> 60,000	2.8%	(6/217)	1.15	(0.45-2.95)	1.06	(0.38-2.95)
Suppressed <0.1 mU/L	Any	5.1%	(9/178)	2.27	(1.11-4.67)	1.97	(0.88-4.43)
	< 20,000	12.5%	(1/8)	8.86	(0.72-109)	*	
	20,000 – 40,000	9.4%	(3/32)	3.82	(1.03-14.2)	4.28	(1.12-16.3)
	40,000 – 60,000	2.6%	(1/38)	1.33	(0.17-10.4)	1.34	(0.17-10.6)
	> 60,000	4.0%	(4/100)	1.62	(0.51-5.09)	1.33	(0.36-4.96)

[a] As compared to the 3rd quintile
** Too small groups for reliable analyses*
Any analyses were adjusted for hCG, gestational age at blood sampling, maternal age, smoking, education level, ethnicity, parity, BMI and fetal gender.
Additional adjustment for hCG and/or exclusion of abnormal gestational age specific hCG values (highest and lowest 1% MoM hCG value) did not change the results.

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CHAPTER 17

THYROID FUNCTION AND PREMATURE DELIVERY IN TPO-ANTIBODY NEGATIVE WOMEN: THE ADDED VALUE OF HCG

Tim I.M. Korevaar, Layal Chaker, Marco Medici, Yolanda B. de Rijke, Vincent W.V. Jaddoe, Theo J. Visser, Eric A.P. Steegers and Robin P. Peeters

Submitted

ABSTRACT

CONTEXT Low maternal thyroid function during pregnancy is associated with premature delivery. hCG is an important stimulator of thyroid function but has a very versatile pattern with high inter and intra-individual variability. We recently showed that thyroid autoimmunity is associated with a lower thyroidal response to hCG stimulation and that this may underlie the higher risk of premature delivery in TPOAb positive women. We hypothesized that also in TPOAb negative women, a lower thyroidal response to hCG stimulation, for example in the setting of isolated TgAb positivity, post-infectious/farmacotoxic or other causes, may be associated with a higher risk of premature delivery and can be identified by an additional hCG measurement.

DESIGN, SETTING, AND PARTICIPANTS TSH, FT4 and hCG concentrations were available in 5644 TPOAb negative women from a population-based prospective cohort study. We tested for interaction between TSH or FT4 and hCG in linear regression models for gestational age at birth and logistic regression models for premature delivery and premature rupture of membranes (PROM). Subsequently, analyses were stratified per TSH percentile increase from TSH ≥85th percentile and hCG was stratified per 10,000 IU/L.

RESULTS The association of TSH with mean gestational age at birth, premature delivery and PROM differed between women with low or high hCG (P for interaction = 0.039, 0.022 and 0.079). Women with a high TSH and low hCG did not have a higher risk of premature delivery or PROM. In contrast, women with a high TSH despite high hCG had 2 to 10-fold higher risk of premature delivery and an up to 4 fold higher risk of PROM.

The association of FT4 with premature delivery did not differ according to hCG. However, for women with very low FT4 (<3rd percentile), a higher hCG was associated with a higher risk of premature delivery (ranging from a 2 to 3.1-fold higher risk; *P*=0.02).

Results remained similar when women with TPOAbs less than the 93rd percentile were excluded.

CONCLUSION In TPOAb negative women, the combination of high hCG with high TSH is associated with a higher risk of premature delivery. We speculate that an adequate thyroidal response to hCG stimulation is important for an uncomplicated pregnancy and that causes other than TPOAb positive thyroid autoimmunity may interfere with this stimulation and are also of clinical relevance in TPOAb negative women. Future studies are needed to replicate these findings and incorporate these results into clinical decision models.

INTRODUCTION

Thyroid hormone (TH) regulates numerous metabolic processes that are important for an uncomplicated pregnancy course. During early pregnancy, human chorionic gonadotropin (hCG) stimulates the TSH receptor which leads to an increase in FT4 concentrations and a subsequent decrease in TSH concentrations.[1] We have recently demonstrated that TPOAb positive women have an impaired response to thyroidal stimulation by hCG.[2] In that study, we also showed that TPOAb positive women with a lower thyroidal response to hCG stimulation have a higher risk of premature delivery. However, even in TPOAb negative women, subgroups of women may have an impaired response to hCG. This is illustrated by studies showing that TPOAb concentrations well below commonly used cut-offs are already associated with a lower thyroid function and a lower thyroidal response (Chapter 13) and by the fact that isolated thyroglobulin antibody (TgAb) positivity is present in up to 25% of women with thyroid autoimmunity.[3] Furthermore, also the presence of TSH receptor blocking antibodies, and other factors including a previous thyroiditis may have caused a lower thyroid functional capacity in TPOAb negative women.

hCG is a major determinant of gestational thyroid function but has a notoriously versatile pattern throughout early pregnancy. hCG is undetectable before conception but after conception hCG rapidly rises to high concentrations until approximately the 9th – 12th week, after which it steadily declines during the remainder of pregnancy.[4] Besides a large inter-individual variability due to differences in gestational age, large differences in hCG concentrations are also present between individuals measured at the same time point.[4] As a consequence, the extent of thyroid stimulation during pregnancy can only be individually quantified through measurement of hCG. Although we recently showed that TPOAb positive women have an impaired thyroidal response to hCG, and that this underlies the increased risk of premature delivery in the these women, it is currently unknown if a suboptimal thyroidal response to hCG stimulation in TPOAb negative women may be of clinical relevance and/or is also a risk factor for premature delivery.

Overt as well as mild forms of maternal thyroid dysfunction have been associated with an increased risk of premature delivery.[5-9] Risk estimates for premature delivery in women with subclinical hypothyroidism and hypothyroxinemia widely differ between studies and vary from a 30% lower risk to a 3.3-fold higher risk.[10-15] It has been hypothesized that these between-study differences are a consequence of variations in population characteristics such as TPOAb positivity, ethnicity, BMI, parity or smoking status.[16-20] Interestingly, such population characteristics are important determinants of hCG concentrations during early pregnancy.[4,21] Moreover, we recently showed that women with subclinical hypothyroidism, but not hypothyroxinemia, have a lower thyroidal response to hCG stimulation regardless of their TPOAb status or BMI.[21]

We hypothesized that even in TPOAb negative women a lower thyroidal stimulation by hCG is associated with a higher risk for premature delivery.

MATERIALS AND METHODS

In order to investigate this hypothesis, hCG concentrations were determined in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, The Netherlands.[22] In this population we have previously reported on the association between TSH or FT4 concentrations and the risk of premature delivery.[23] In the same population, we investigated whether the addition of hCG would improve the interpretation of maternal TSH and FT4 concentrations during early pregnancy

for the risk assessment of premature delivery.[23] Population for analyses, covariates and definitions of outcomes have been described previously.[23] In short, 6264 pregnant women during early pregnancy had data available on TSH, FT4 or TPOAb concentrations and gestational age at birth. We excluded women with twin pregnancies (N=128), preexisting thyroid disease or thyroid interfering medication usage (N=89), women that underwent fertility treatment (N=76), TPOAb positive women (N=312) and women with hCG measurement that were not performed within the same week as thyroid function measurement (N=15). Premature delivery was defined as a gestational age at birth of <37 weeks, spontaneous premature delivery was defined as not having had a delivery after induction of labor or by an elective caesarean section. Premature rupture of membranes (PROM) was defined as ruptured membranes before 37 weeks' gestation. All analyses on premature delivery were adjusted for maternal age, BMI, smoking, parity, education level, ethnicity, height and fetal gender.

Statistical analysis

To fulfill model assumptions TSH values were logarithmically transformed. We used linear or logistic regression models utilizing restricted cubic splines with 3 knots to assess non-linearity of the associations between TSH, FT4, hCG and gestational age or (very/spontaneous) premature delivery and PROM. Subsequently, linear or logistic regression models were built accordingly. In order to test the hypothesis that high hCG helps to identify true subclinical hypothyroidism and/or true hypothyroxinemia, we stratified the association between high TSH and/or low FT4 concentrations and premature delivery according to hCG. To investigate if the association between continuous TSH and/or FT4 concentrations and premature delivery or PROM would differs according to hCG concentrations, we tested for interaction by adding a product term of TSH or FT4 and hCG to the model. The identification of clinically relevant effect modification requires more statistical power, therefore, we considered interaction terms with a *P*-value of <0.15 for assessment of clinical relevance by subsequently stratifying analyses. We performed a similar interaction analysis in which hCG was replaced with gestational age at blood sampling because gestational age is considered as a marker of hCG concentrations and is therefore used in other clinical studies on gestational thyroid function. Removal of outliers for TSH, FT4 or hCG did not change the results. For variables with missing data, multiple imputation according to the Markov Chain Monte Carlo method was used [24]. Five imputed data sets were created and pooled for analyses. For the prematurity database, TSH, FT4, hCG, smoking, socio-economic status, ethnicity, parity, BMI, fetal gender and gestational age at blood sampling were added to the model (missing due to non-response/non-recording in 6.3%, 5.7%, 3.5%, 12.8%, 7.2%, 5.7%, 1.9% and all other variables <2.0%, respectively). Furthermore, we added maternal age, total T4 and TPOAb concentrations as prediction variables only. No significant differences in descriptive characteristics were found between the original and imputed datasets. All statistical analyses were performed using R statistical software v3.03 (package *rms*, *visreg*), or Statistical Package of Social Sciences version 20.0 for Windows (SPSS Version 22.0. Armonk, NY: IBM Corp).

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RESULTS

The final study population comprised 5644 women, descriptive characteristics of which are shown in Table 1. The association of TSH with mean gestational age at birth, premature delivery and PROM differed between women with low or high hCG (*P*interaction = 0.039, 0.022 and 0.079, respectively; Supplemental Table 1). Figure 1 displays heatmaps that graphically illustrates the differences in mean gestational age (A), the risk of premature delivery (B) and the risk of PROM (C) according to the

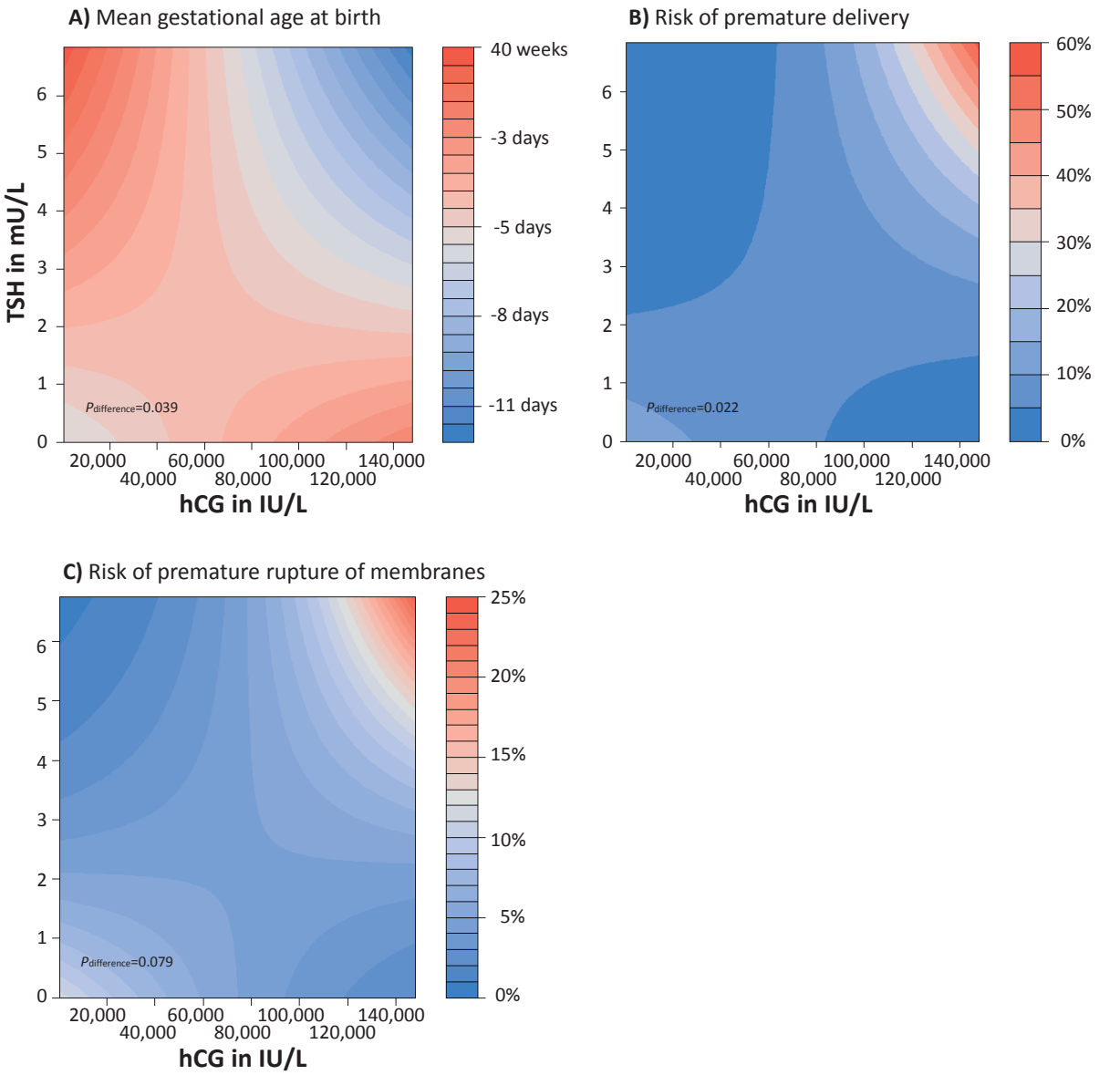
combination of hCG and TSH in the whole study population (blue indicates low, and red indicates high mean gestational age, risk of premature delivery or PROM).

TABLE 1. *Descriptive statistics of the study population.*

		Median	(95% range)
Median TSH	(mU/L)	1.33	(0.05-4.13)
Median FT4	(pmol/L)	14.8	(10.3-22.3)
Median hCG	(IU/L)	44,625	(11,999-106,339)
Median UIC*	(µg/g)	273	(96-777)
Premature delivery	(<37 wks)	276	(4.9)
Gestational age[a]		13.4	(9.6-17.6)
Maternal age[d]		30.3	(19.5-38.8)
Maternal height[e]		168	(153-182)
Maternal BMI		23.5	(18.5-38.8)
Parity[c]			
0		3226	(57.2)
1		1681	(29.8)
≥2		738	(13.1)
Smoking[c]			
Non-smokers		4140	(73.4)
Stopped smokers		506	(9.0)
Smokers		997	(17.7)
Education level[c]			
Low		606	(10.7)
Middle		2599	(46.0)
High		2439	(43.4)
Ethnicity[c]			
Dutch		2803	(51.6)
Moroccan		341	(6.3)
Turkish		455	(8.4)
Surinamese		479	(8.8)
Other western		491	(9.0)
Other non-western		866	(15.9)
Child gender[c] (boys %)		2853	(50.5)

** Based on data in a subset of N=1031 women*
[a] *Data shown as n (%)*
[b] *At time of blood sampling; Data shown in weeks*
[c] *Data shown as n (%)*
[d] *Data shown as median in years*
[e] *Data shown in centimeters*

FIGURE 1. *Mean gestational age at birth and the risk of premature delivery according to TSH and hCG levels.*



Mean gestational age at birth (A), the risk of premature delivery (B) and the risk of premature rupture of membranes (C) according to TSH and hCG levels. Heat maps show hCG on the x-axis, TSH on the Y-axis and the mean gestational age of risk of premature delivery on the Z-axis which ranges from low values or risks (blue) to the high levels or risks (red).

In order to quantify the differences in risks, we stratified the association of high TSH (per percentile) with premature delivery for hCG (per 10,000 IU/L; Table 2). Women with a high TSH and low hCG did not have a higher risk of premature delivery, and the majority of effect estimates pointed towards a protective effect (Figure 1B and Table 2). In contrast, women with a high TSH despite high hCG had 2 to 10-fold higher risk of premature delivery depending on the cut-offs used (Figure 1 and Table 2).

A similar analysis for PROM showed that women with an high TSH and low hCG did not have a higher risk of PROM, and the majority of effect estimates pointed towards a protective effect (Figure 1C and Table 3). In contrast, women with an high TSH despite high hCG had a risk of PROM that ranged between a protective effect up to a 4-fold higher risk depending on the cut-offs used (Figure 1C and Table 3).

TABLE 2. *Outline of the interaction between hCG and TSH in the risk of premature delivery.*

		hCG level							
TSH percentile (value in mU/L)	**Overall**	**<20,000**	**20,000-30,000**	**30,000-40,000**	**40,000-50,000**	**50,000-60,000**	**60,000-70,000**	**70,000-80,000**	**>80,000**
≥ 96 (4.02)	**1.88 (1.14-3.12)**	*	*	*	0.74	1.54	3.42	5.91	9.96
≥ 95 (3.72)	1.62 (0.98-2.67)	0.64	*	0.52	0.57	1.15	2.34	4.46	6.84
≥ 94 (3.49)	1.50 (0.95-2.35)	0.46	*	0.53	0.83	1.06	1.93	2.77	6.29
≥ 93 (3.32)	1.40 (0.92-2.12)	0.40	0.41	0.42	0.73	0.88	1.82	2.48	5.64
≥ 92 (3.15)	1.28 (0.85-1.92)	0.33	0.38	0.61	0.60	0.67	1.67	2.16	5.05
≥ 91 (3.01)	1.18 (0.79-1.74)	0.53	0.42	0.54	1.00	1.00	1.60	2.20	4.02
≥ 90 (2.91)	1.24 (0.85-1.80)	0.48	0.39	0.49	0.93	0.95	1.48	3.36	3.33
≥ 89 (2.81)	1.16 (0.80-1.69)	0.51	0.37	0.44	0.87	0.89	1.42	2.41	3.05
≥ 88 (2.73)	1.23 (0.87-1.75)	0.61	0.34	0.39	0.79	0.82	2.60	3.79	2.68
≥ 87 (2.66)	1.22 (0.85-1.74)	0.80	0.38	0.39	0.88	1.08	2.39	2.59	2.56
≥ 86 (2.59)	1.16 (0.82-1.64)	0.72	0.50	0.36	0.86	0.91	2.07	2.59	2.29
≥ 85 (2.53)	1.10 (0.77-1.56)	0.89	0.48	0.35	0.96	0.83	1.89	2.22	1.85

** None of the women in this subgroup had a premature delivery.*
Table 2 shows the adjusted odds ratio for premature delivery (<37 weeks) according to different percentile cut-offs for TSH, stratified by concomitant hCG levels. All analyses were adjusted for maternal age, smoking, education level, ethnicity, parity, BMI, height and fetal gender. hCG cut-off groups were not associated with the risk of premature delivery.

TABLE 3. *Outline of the interaction between hCG and TSH in the risk of premature rupture of membranes.*

		hCG level						
TSH percentile (value in mU/L)	**Overall**	**<20,000**	**20,000-30,000**	**30,000-40,000**	**40,000-50,000**	**50,000-60,000**	**60,000-70,000**	**>70,000**
≥ 96 (4.02)	1.24 (0.60-2.55)	*	*	1.48	0.70	1.31	1.63	4.20
≥ 95 (3.72)	1.10 (0.54-2.25)	*	*	1.11	0.62	1.14	1.13	4.31
≥ 94 (3.49)	0.94 (0.47-1.89)	*	*	0.90	0.55	0.85	0.96	3.33
≥ 93 (3.32)	0.97 (0.51-1.84)	*	*	0.80	1.13	0.75	0.78	3.24
≥ 92 (3.15)	0.90 (0.51-1.62)	*	*	0.71	0.96	0.62	0.76	3.83
≥ 91 (3.01)	0.81 (0.47-1.43)	*	0.08	0.70	0.87	0.52	0.68	3.44
≥ 90 (2.91)	0.84 (0.50-1.44)	*	0.09	0.60	0.95	0.54	0.65	3.74
≥ 89 (2.81)	0.82 (0.48-1.40)	*	0.01	0.75	0.82	0.50	0.62	3.54
≥ 88 (2.73)	0.90 (0.55-1.46)	0.42	0.01	0.68	0.91	0.46	1.55	3.36
≥ 87 (2.66)	0.97 (0.59-1.59)	0.61	0.39	0.64	0.87	0.69	1.40	3.38
≥ 86 (2.59)	0.94 (0.57-1.53)	0.58	0.39	0.81	0.81	0.61	1.32	3.25
≥ 85 (2.53)	0.91 (0.55-1.49)	0.79	0.36	0.77	0.84	0.55	1.22	2.92

** None of the women in this subgroup had a premature delivery; Too little PROM occurred to do reliable analyses for a cut-off for hCG of >80,000 IU/L.*
Table 3 shows the adjusted odds ratio for premature rupture of membranes (<37 weeks) according to different percentile cut-offs for TSH, stratified by concomitant hCG levels. All analyses were adjusted for maternal age, smoking, education level, ethnicity, parity, BMI, height and fetal gender. hCG cut-off groups were not associated with the risk of premature rupture of membranes.

In the whole population, the association of FT4 with premature delivery did not differ between low and hCG (*P*interaction were 0.52, 0.69 and 0.68 for mean gestational age at birth, premature delivery and PROM, respectively; Supplemental Table 1). However, for women with very low FT4 (at or below the 3rd percentile) the risk of premature delivery differed between low and high hCG, ranging from a 2 to 3.1-fold higher risk (OR 95%CI for whole group 2.34 (1.32-4.14); interaction term hCG groups and FT4 ≤3rd percentile *P*=0.02). However, for women with very low FT4 (at or below the 3rd percentile), the risk of PROM did not differ between low and high hCG, ranging from a 2.4 to 2.5-fold higher risk (OR 95%CI for whole group 2.52 (1.29-4.93)).

Similar results were obtained when TPOAb positivity was defined by a lower cut-off (e.g. <10 IU/L), after exclusion of women with preeclampsia or when analyses were restricted to women with a spontaneous delivery. hCG concentrations were not associated with gestational age at birth, premature delivery or PROM. The association of TSH or FT4 with premature delivery did not differ by gestational age at blood sampling, used as a potential proxy for hCG.

DISCUSSION

In th e current study, we demonstrate that also in TPOAb negative women the association of maternal thyroid function during pregnancy with the risk of premature delivery is modified by hCG concentrations. Our main finding is that women with high TSH despite high hCG have a 2 to 10-fold higher risk of premature delivery and a 1.6 to 4.2-fold higher risk of PROM. In contrast, women with high TSH and low hCG did not have a higher risk of premature delivery. We also demonstrate that women with very low FT4 and high hCG had a higher risk of premature delivery, but not PROM, as compared to women with very low FT4 and low hCG.

We have previously shown that the thyroidal response to hCG stimulation is considerably impaired in TPOAb positive women and that only TPOAb positive women with a low thyroidal response to hCG have a higher risk of premature delivery.[2] The results of the current study in TPOAb negative women further strengthen the concept that a lower thyroidal response to hCG stimulation increases the risk of premature delivery. It is still likely that the subset of TPOAb negative women with a lower thyroidal response to hCG stimulation have a form of thyroidal autoimmunity such as isolated TgAb positivity or the presence of blocking TSH receptor antibodies. In addition, the presence of a subclinical form of thyroid autoimmunity would fit with recent data from three Dutch birth cohorts demonstrating that TPOAb concentrations well below commonly used cut-offs are associated with lower thyroid function and a lower thyroidal response to hCG stimulation.[21] However, the results in the current study were not altered after excluding women with very low TPOAb concentrations. (e.g. <10 IU/L). Alternatively, other (subclinical) forms of systemic disease or a natural variation in thyroid functional capacity may be the underlie the findings of the current study. Further studies are required to investigate risk factors for a lower thyroid functional capacity in TPOAb negative women.

Our results suggest that measurement of hCG concentrations during pregnancy may improve the interpretation of (high) TSH concentrations, while it is less likely to improve the interpretation of FT4 concentrations. The discrepancy between our results on TSH and FT4 may be explained by the fact that for absolute concentrations of TSH the relative change during pregnancy is larger than the relative change in absolute FT4 concentrations given the log-linear relationship between FT4 and TSH. Higher TSH concentrations both reflect TH shortage as well as counteract thyroid hormone shortage. Intriguingly, in a previous paper, our group demonstrated that the FT4 response to hCG may be of relevance to determine the risk in TPOAb positive women.[2] In that study, TPOAb positive women had much higher

TSH concentrations overall, and the change in TSH may thus be less specific for the thyroidal response than in TPOAb negative women that have much lower TSH concentrations. Although both of these studies require replication, the results from the current study on the combination of high TSH and high hCG also indicate that a deviation from the naturally occurring physiology, i.e. a suboptimal thyroid response to hCG stimulation, is a risk factor for premature delivery.

The risk of premature delivery in women with subclinical thyroid dysfunction has been investigated in many different populations. Taken together, these studies report a wide range in the risk of premature delivery ranging from a 30% decrease to a 3.3-fold increased risk.[10-15] The results from our study suggest that the large between-study differences in the risk of premature delivery for women with subclinical thyroid dysfunction may be due to between-study differences in the thyroidal response to hCG stimulation or absolute hCG concentrations at time of thyroid function measurement. Unfortunately, none of the previous studies that investigated the association of maternal thyroid function with premature delivery have hCG measurements available. Although gestational age at blood sampling may be a proxy for hCG, we could not identify a difference in the association of TSH or FT4 with premature delivery according to a different gestational age at blood sampling. Furthermore, we were unable to replicate the results of other studies by only selecting subjects with the same gestational age as in other studies, respectively 10-13 weeks, 10 3/7 - 13 6/7 weeks and a mean of 14.1 weeks.[10,15,25] This suggests that between-study differences in the gestational age at intake do not underlie the large between-study differences in the risk of premature delivery in women with low thyroid function. Furthermore, gestational age at intake is unlikely to be a good proxy for hCG given the large inter-individual differences in hCG concentrations. Future studies are needed to verify if the observed differences in risk estimates between populations are due to differences in population hCG concentrations or differences in thyroidal stimulation.

TSH and FT4 concentrations are partly a reflection of hCG concentrations and/or differences in hCG isoforms. Therefore it is possible that the results from the current study are a reflection of the effects of hCG on premature delivery since high concentrations of hCG (standardized to gestational age) have been associated with a higher risk of premature delivery.[26-28] However, in the current study absolute hCG concentrations were not associated with the risk of premature delivery. We demonstrate that only combinations of high TSH or very low FT4 with high hCG were associated with a higher risk of premature delivery, a combination that is suggestive of a suboptimal thyroidal response to hCG stimulation. Interestingly, the assay used to measure hCG concentrations in the current study measures total hCG concentrations, including its various isoforms such as nicked, asialo and hyperglycosylated hCG. It has been shown that hCG subtypes have a different thyrotropic activity.[29-31] Although in pathological conditions such as preeclampsia different ratios of hCG isoforms have been reported,[32,33] it is unknown if certain hCG isoforms are associated with premature delivery.

In conclusion, we show that the risk of premature delivery according to TSH concentrations is modified by hCG concentrations. Of all TPOAb negative women with high-normal TSH concentrations, only women with high hCG concentrations at the time of TSH measurement had a higher risk of premature delivery. These data suggest that the assessment of maternal thyroid function together with hCG concentrations can improve the risk assessment of premature delivery and give new insights into the pathophysiology of the association between maternal thyroid function and premature delivery. When further replicated, this concept may improve clinical practice by allowing clinicians to better identify women at risk for pregnancy complications.

APPENDIX

SUPPLEMENTAL TABLE 1. *Sensitivity analyses investigating interaction between TSH or FT4 and hCG for premature delivery.*

	Gestational age at birth	Premature delivery	Premature Rupture of Membranes
	P-value	***P-value***	***P-value***
TSH	0.57	0.22	0.16
hCG	0.20	0.09	0.19
TSH * hCG	0.039	0.022	0.079
FT4	0.021	0.40	0.73
hCG	0.20	0.09	0.19
FT4 * hCG	0.52	0.69	0.68

Supplemental Table 1 shows the P-values for the linear association between TSH, hCG or their product interaction term and mean gestational age at birth, premature delivery (<37 weeks gestation) and premature rupture of membranes (<37 weeks). All analyses were adjusted for maternal age, smoking, education level, ethnicity, parity, BMI, height and fetal gender. These results were similar for very premature delivery (<34 weeks) an in women with spontaneous premature delivery only. We used P=0.15 as a cut-off for subsequent stratification analyses.

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PART 5: GENERAL DISCUSSION AND SUMMARY

CHAPTER 18

THYROID DISEASE IN PREGNANCY: NEW INSIGHTS IN DIAGNOSIS AND CLINICAL MANAGEMENT

Tim I.M. Korevaar, Marco Medici, Theo J. Visser and Robin P. Peeters

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ABSTRACT

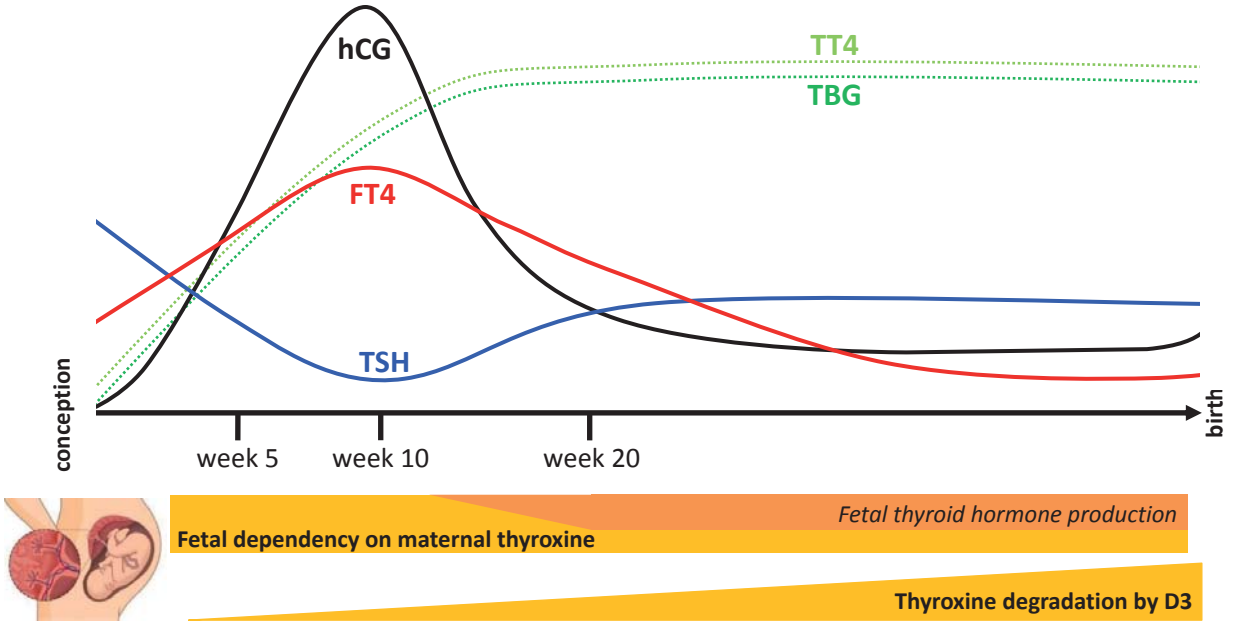
Adequate thyroid hormone availability is important for an uncomplicated pregnancy course and optimal fetal growth and development. Overt thyroid disease is associated with a wide range of adverse obstetric and child development outcomes, and an increasing number of studies now also indicate that milder forms of thyroid dysfunction are also associated with these adverse outcomes. The definition of both overt as well as subclinical thyroid dysfunction has changed considerably over the last few years as new data indicate that the commonly used fixed upper TSH limits of 2.5 or 3.0 mU/L are too low. Furthermore, recent studies have also shown that reference ranges are not necessarily the best cut-off to identify pregnancies at high risk of adverse outcomes and particularly thyroid autoimmunity and hCG seem to play a more prominent role than previously thought. There is still a lack of data on the effects of treatment. However, new data indicate that we should also be aware of the potential for overtreatment with levothyroxine. This review will put recent data on reference ranges, determinants, risk of adverse outcomes and treatment options into perspective and provides an overview of the current views on thyroid physiology during pregnancy as well as identification of high-risk individuals that may benefit from levothyroxine treatment.

INTRODUCTION

Thyroid hormone (TH) is essential for a normal pregnancy and fetal development. The fetal thyroid is not functionally matured until week 18-20.[1] During early pregnancy, the fetus therefore predominantly depends on the supply of maternal TH. As a consequence, untreated maternal hypothyroidism during pregnancy is associated with an increased risk of adverse pregnancy as well as child outcomes.[2]

Pregnancy has clear effects on thyroid physiology. Maternal supply of TH to the fetus, increasing levels of thyroxine-binding globulin (TBG), increasing iodine clearance, and degradation of TH by placental type 3 deiodinase (D3) all necessitate an increase in TH production to ensure adequate TH availability (Figure 1).[2] High levels of the pregnancy hormone human chorionic gonadotrophin (hCG), a weak agonist of the TSH receptor, increase TH production by stimulating the thyroid (Figure 1).[3] These pregnancy-specific changes and increased demand may expose pre-existing mild thyroid dysfunction as gestational thyroid disease.

FIGURE 1. *Pregnancy-specific changes in thyroid physiology.*



Overt maternal hypothyroidism (elevated TSH with low FT4) during pregnancy occurs in 0.2 to 0.6% of pregnant women.[2,4,5] Subclinical maternal hypothyroidism (elevated TSH with normal FT4) is even more prevalent, occurring in 3.5 to >18% of all pregnancies, depending on the definition used.[6-10] The main risk factor is autoimmune thyroid disease, with TPO-autoantibody (TPOAb) positivity occurring in roughly one third of women.[11,12] Gestational hyperthyroidism (biochemically defined by elevated FT4 and suppressed TSH) is much more frequent and diagnosed in about 1-3% of pregnancies and most often secondary to high hCG concentrations. In about 50% of cases, it is associated with hyperemesis gravidarum.[13] Pathological hyperthyroidism (predominantly Graves' disease and toxic nodules or goiter) during pregnancy is less frequent and has a frequency of 0.4-1% before pregnancy and approximately 0.2% during pregnancy.[13]

In recent years, evidence has accumulated on the negative consequences of maternal subclinical hypothyroidism, hypothyroxinemia (low FT4 with normal TSH) and thyroid autoimmunity on pregnancy

outcomes and child development. Although the risks of adverse outcomes is considerably lower compared to overt disease, the higher prevalence of subclinical thyroid disease makes it an important public health issue. However, there has been a continuous debate on the definition of these disease entities, limiting comparability between studies and definition of treatment cut-offs. Also (subclinical) hyperthyroidism has been associated with negative pregnancy outcomes, and recent data suggest that high concentrations of maternal FT4 may be equally detrimental as low concentrations.[14] However, since recent studies have shown that antithyroid drugs are associated with potential harms as well, treatment options are limited. Given these complexities, it remains challenging how and when to evaluate for thyroid dysfunction, and when and how to treat thyroid illness during pregnancy. This review aims to put the results of recent studies in the field of thyroid and pregnancy into perspective and discuss how recent findings may impact clinical decision making.

BIOCHEMICAL DEFINITION OF THYROID DYSFUNCTION

Because of the major changes in thyroid physiology, the definiton of thyroid disease during pregnancy is best defined according to pregnancy-specific reference ranges calculated in pregnancies free of thyroid function interfering factors.[7,9,15,16] The latter would require that women with major disease, thyroid autoimmunity (in particular TPOAb positivity), thyroid (interfering) drug use, twin pregnancies and/or IVF treatment are excluded. The importance of such exclusions is illustrated by a study of Lambert-Messerlian *et al.* demonstrating that TSH upper limits changed from 4.15 to 3.37 mU/L and from 3.77 to 3.35 mU/L in the first and second trimester after exclusion of TPOAb positive women.[17]

It is often mentioned that the minimum number of samples required for reference range calculations is 120. However, this number is the minimum for calculation of a 90% reference range of a normally distributed measurement outcome.[4,18,19] The number of serum samples needed to adequately define reference ranges (based on the 2.5^{th}-97.5^{th} percentiles) for measurements with a skewed distribution (e.g. TSH and to a lesser extent FT4) is approximately 400.[4,18,19] For centers unable to calculate their own reference ranges, previous international guidelines additionally provided reference ranges for TSH, recommending fixed upper limits of 2.5 and 3.0 mU/L for the first and second/third trimesters, respectively.[7,9,15] However, more recent studies have consistently shown that the use of such upper limits leads to overdiagnosis of subclinical hypothyroidism, as between 8% to 28% of different populations have a TSH above these fixed cut-offs.[20,21] In addition, the TSH concentrations from which a lowering in FT4 starts to occur (as a proxy for mild thyroid failure) likely lies between 4 to 5 mU/L.[21] When summarizing 14 recent studies that report population-based reference ranges encompassing over 65.000 individuals, more than 90% of all studies that calculated population-based reference ranges report an upper limit for TSH that is (considerably) higher than the proposed 2.5 and 3.0 mU/L cut-offs.[4]

Given these novel insights, the 2016 draft guidelines of the American Thyroid Association advocate the use of pregnancy-specific, population-based reference ranges. When these are unavailble, it is recommended to adopt population-based reference ranges that have been determined using the same assay and in a population with similar characteristics. An overview of adequately sized studies from different populations, using various assays, is provided in the Supplementary Materials. Finally, if none of the published studies are generalizable to the population of interest, it is advocated to use a fixed upper limit for TSH of 4 mU/L for TSH (similar to the upper limits in large studies from iodine sufficient populations).

Despite the dominant role of TSH in defining gestational thyroid dysfunction, a (subsequent) FT4 measurement is necessary to distuinguish between overt and subclinical thyroid disease. Commonly used FT4 immunoassays have been reported to be less accurate due to high concentrations of TH binding proteins during pregnancy, particulary in the third trimester.[22-25] Fortunately, the majority of thyroid function tests take place in the first half of pregnancy. Moreover, immunoassays are still useable because the correlation between FT4 concentrations measured using immunoassays before or after equilibrium dialysis is high.[23,26,27] Because of this inter-assay correlation, the calculation of pregnancy-specific, population-based (thus also assay specific) reference ranges will adequately identify women with true low, or high, FT4 concentrations and makes relevant misclassification unlikely.[28] It has been postulated that total T4 (TT4) concentrations may be used to assess thyroid dysfunction during pregnancy by raising the lower limit of the non-pregnancy reference range to 150%.[24] However, since more than 99% of TT4 is bound to thyroid hormone binding proteins, this seems like a crude estimate of the available fraction of T4. Since TT4 is highly dependent on changes in TBG, TT4 has a larger variability during early pregnancy than FT4.[29,30] Furthermore, TT4 explains much less of the variation in TSH than FT4 (2.5% for TT4 versus 8.0% for FT4), and therefore seems a lesser reflection of the HPT axis.[29] During early pregnancy, only FT4, but not TT4, is associated with adverse pregnancy and child outcomes.[30,31] Taken together, these data suggest that FT4 is a (more) useful thyroid function measurement during early pregnancy.[30]

DETERMINANTS OF THYROID (DYS)FUNCTION DURING PREGNANCY

The identification and quantification of determinants of thyroid function adds to our knowledge on (patho)physiology, can improve the interpretation of thyroid function measurements and could potentially be used for identification of individuals at high-risk for thyroid dysfunction. Recent studies on determinants have further quantified the importance of well-known risk factors such as iodine, patient characteristics (including body mass index (BMI) and ethnicity) as well as hCG and other placental factors.

Iodine is a major component of TH and essential for its production. Severe maternal iodine deficiency leads to overt hypothyroidism and offspring cretinism.[32] Although studies have predominantly focused on the consequences of low iodine status on thyroid function, also high-normal iodine intake is associated with lower thyroid function.[33] Shi *et al.* demonstrated that the group of women with relatively high urinary iodine concentrations (UIC; >250 µg/L) had an up to 2.2-fold higher risk of subclinical hypothyroidism and an up to 2.9-fold higher risk of hypothyroxinemia.[33] Although low UIC (e.g. <100 µg/L) was associated with a higher risk of thyroid autoimmunity (both TPOAb positivity and TgAb positivity) and overt hypothyroidism, there was no association with subclinical hypothyroidism or hypothyroxinemia.[33]

Ethnic differences between, as well as within, populations are associated with maternal thyroid function during pregnancy.[34-38] Although ethnicity is a composite of genetic, dietary and cultural differences, this illustrates why population-based reference ranges differ throughout the world.[38,39]

BMI has consistently been shown to be a determinant of thyroid function during pregnancy.[40-45] Higher BMI is associated with higher TSH concentrations,[41-45] lower FT4 concentrations[41-45] and higher FT3 concentrations[41,43] (and a higher T3/T4 ratio). A study by Männistö et al. showed that cut-offs for TSH and FT4 differ between women with a BMI of 20-24.9 or >30 kg/m^2 (for example for high TSH: 2.86 versus 3.50 mU/L).[43] Although various other characteristics such as maternal age and smoking have

been identified as risk factors for thyroid dysfunction, a combination of these clinical characteristics does not accurately predict the risk of thyroid dysfunction in the general population.[46]

The rapid rise in hCG concentrations during early pregnancy increase FT4 concentrations which subsequently leads to a decrease in TSH concentrations.[29,47,48] Factors that affect this thyroidal response to hCG are more likely pregnancy-specific determinants of thyroid function, rather than general determinants of thyroid function (i.e. determinants as in non-regnant states), determinants of thyroid function. Recent studies from our group have shown that the thyroidal response to hCG stimulation is severely impaired in women with thyroid autoimmunity, as reflected by TPOAb positivity.[48] Other factors that may also reduce the thyroidal response to hCG stimulation are a higher BMI, and to a lesser extent a higher parity (specifically ≥2) and male fetal sex.[12]

Besides hCG, the placenta produces angiogenic factors such as anti-angiogenic soluble FMS-like tyrosine kinase (sFlt1) and placental growth factor (PlGF).[49] The thyroid has a high vascular density and animal studies demonstrate that changes in these factors can inhibit thyroid vasculature density by up to 68%.[50,51] The first study in human pregnancies showed that high sFlt1 concentrations are associated with a 2.4-fold higher risk of subclinical hypothyroidism and a 3-fold higher risk of hypothyroxinemia while high PlGF concentrations were associated with a 1.8-fold higher risk of hypothyroxinemia.[52] Furthermore, these factors were also shown to influence the thyroidal response to hCG stimulation.[52] These data provide novel insights into the pregnancy-specific thyroid physiology and illustrate potential pathways through which the placenta may influence thyroid function other than via hCG.

Although various studies show that clinical characteristics affect reference ranges between, or even within populations, they are poor predictors of overt thyroid dysfunction. It therefore remains to be elucidated whether the implementation of trimester, BMI or ethnicity-based reference ranges are more specific to identify gestational thyroid disease. Future studies should aim to identify novel determinants and further unravel known determinants (i.e. genetics, endocrine disruptors) to allow identification of clinically relevant patient subgroups.

CONSEQUENCES AND TREATMENT OF THYROID DISEASE

Overt hypothyroidism

Overt maternal hypothyroidism has consistently been associated with a higher risk of adverse pregnancy complications including premature delivery, low birth weight, miscarriage, pre-eclampsia,[53] as well as detrimental effects on fetal neurodevelopment.[54] A large case-control study demonstrated a seven-point reduction in (IQ) among children born to untreated hypothyroid women when compared to euthyroid controls.[54] These children also had a delay in motor skill development, language development and attention at 7-9 years of age.[54] Interestingly, these deficits were not observed in children born from mothers who received levothyroxine treatment later in pregnancy.[54] Similarly, there are no data that suggest that women with adequately treated hypothyroidism have an increased risk of pregnancy complications, which is in sharp contrast to untreated hypothyroidism. There are no randomized controlled trials of levothyroxine treatment for overt hypothyroidism during pregnancy. However, it is deemed unethical to perform a placebo controlled trial given the large effects of over hypothyroidism on various adverse outcomes and the general consensus is that overt hypothyroidism during pregnancy should be treated as early as possible.[(ref guideline)] Considering the important role of maternal T4 for fetal brain development (via local T4 to T3 conversion in the fetal brain), levothyroxine is the treatment of choice. Patients using T4 and T3 combination therapy or dessicated thyroid extracts often have a lower

than physiological T4/T3 ratio, and may therefore be at risk to have insufficient transfer of maternal T4 to the fetal brain.[55-58]

Subclinical hypothyroidism

Similar to overt hypothyroidism, subclinical hypothyroidism has been associated with a higher risk of pregnancy loss, placental abruption, premature delivery, pre-eclampsia and neonatal death.[59-61] However, in these studies subclinical hypothyroidism has been defined differently (non-prenancy reference ranges, fixed TSH cut-offs or calculated pregnancy specific reference ranges).[60] Although subclinical hypothyroidism is associated with various adverse pregnancy outcomes overall, the most specific associations are found when defined according to population-based reference ranges.[4,60,61]

Thyroid autoimmunity is a major risk factor for subclinical hypothyroidism.[20] Approximately one third of all women with subclinical hypothyroidism are TPOAb positive.[11,12] Recent studies have shown that the combination of subclinical hypothyroidism with TPOAb positivity is associated with a higher risk of adverse outcomes such as miscarriage, gestational diabetes and premature delivery.[62-65] Although the risk of adverse pregnancy outcomes is much lower in women with subclinical hypothyroidism that are TPOAb negative, it is still higher than in euthyroid women.[62-65] However, it seems that the combined presence of high TSH and TPOAb positivity leads to a synergistically higher risk of adverse pregnancy outcomes and that this higher risk is already present for high-normal TSH concentrations (i.e. for TPOAb positive women with TSH >2.5 mU/L).[62-65]

There is a lack of data on the effects of treatment for subclinical hypothyroidism. A recent observational study by Maraka *et al*. found that treatment of subclinical hypothyroidism (defined by a TSH between 2.5-10 mU/L, FT4 >0.8 ng/dL and/or total thyroxine >7.5 mcg/dL) with a median dosage of 50µg (IQR 25-62.5) was associated with lower pregnancy loss (10.6% vs. 13.5%; OR 0.62 [0.48-0.82]) but a higher rate of premature delivery (7.1% vs. 5.2%; OR 1.60 [1.14-2.24]), but no differences in the risk of preterm labor or preterm rupture of membranes. Also, differences were identified for gestational diabetes (12.0% vs. 8.8%; OR 1.37 [1.05-1.79]) and pre-eclampsia (5.5% vs. 3.9%; OR 1.61 [1.10-2.37]). Interestingly, the beneficial effect of treatment was dependent on the TSH concentrations at presentation.[66] This was illustrated by subsequent stratification revealing that the beneficial effect on pregnancy loss was predominantly present in women with TSH concentrations above 4.0 mU/L (OR 2.5-4.0 mU/L: 0.87 [0.62-1.22], vs >4.0 mU/L: OR 0.43 [0.29-0.63]; *P* for difference <0.01). Unfortunately, there were no data available on TPOAbs. Still, these data suggest that there is no benefit of levothyroxine treatment in women with high-normal TSH (roughly a TSH between 2.5-4.0 mU/L). Further studies are required to identify the threshold TSH concentration from which treatment becomes beneficial. In contrast to the consistent findings on pregnancy outcomes, subclinical hypothyroidism has not been associated with offspring neurodevelopment outcomes. Studies investigating the effects of levothyroxine treatment in women with subclinical hypothyroidism on offspring neurocognition are discussed in the section on hypothyroxinemia.

Thyroid autoimmunity

As a reflection of thyroid autoimmunity, TPOAbs are the most important risk factor for thyroid dysfunction during pregnancy. It has consistently been shown that TPOAb positive women have higher TSH concentrations, lower FT4 concentrations and also a higher risk of thyroid dysfunction during pregnancy.[20,67] Intriguingly, TPOAb positivity in itself is associated with a higher risk of miscarriage and premature delivery.[64,67,68] Two major hypotheses on the underlying mechanisms through which TPOAb positivity may increase the risk of adverse outcomes have been postulated: (1) Women with a higher susceptibility to autoimmunity in general are more likely to be TPOAb positive [50,51] and may also have a

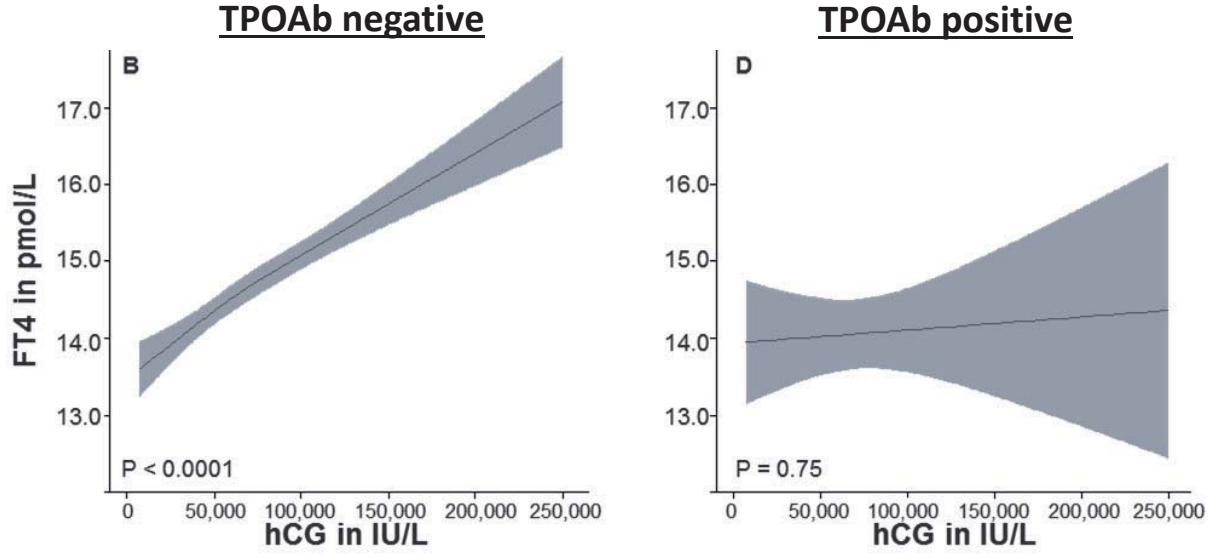
higher risk of adverse pregnancy outcomes, creating a spurious association for TPOAb positivity.[69,70] (2) TPOAb positivity may lead to (mild) thyroid dysfunction and this subsequently leads to a higher risk of adverse pregnancy outcomes.

Although various arguments can be applied to this hypothesis, two clinical trials have shown that levothyroxine treatment in TPOAb positive women markedly decreases the risk of miscariage and/or premature delivery.[52,53] Negro et al. randomized 115 TPOAb positive women to receive levothyroxine at a mean gestational age of 10 weeks with a dosage based on their TSH (<1 mU/L: 0.5μg/kg·d; 1-2 mU/L: 0.75 μg/kg·d; >2 mU/L or TPOAbs >1500 kIU/liter: 1 μg/kg·d).[71] In this study, levothyroxine treatment markedly reduced the rate of miscarriage (from 13.8% versus 3.4%) and of premature delivery (22.4% versus 7.0%).[71] In this study, the first endocrinological visit on average took place in the 11th week of pregnancy and 92% of women visited the endocrinologist before the 20th wk of gestation. Given that miscarriage is defined by pregnancy loss before the 20th week of gestation, and roughly 80% of miscarriages occur before the 12th week of gestations, the design of the trial was not adequate to study this outcome. Using the same protocol, Nazarpour *et al.* randomized 131 TPOAb positive women to receive levothyroxine at a mean gestational age of 11 weeks.[12] This study also found that levothyroxine reduced the rate of premature delivery (23.7% versus 7.21%) but there was no beneficial effect of levothyroxine on the rate of miscarriage (3.6% versus 3.4%).[66] Both groups had rather high TSH concentrations (3.7 mU/L (IQR 2.4-4.8) and 3.2 mU/L (2.1-5.2) at presentation, likely explaining the high rate of premature delivery in the non-treated group. Although both trials have limitations, these findings strongly suggest that the adverse outcomes associated with TPOAb positivity are indeed effectuated through changes in thyroid function.[71,72]

Further evidence from ongoing RCTs investigating levothyroxine treatment in TPOAb positive women is awaited [TABLET trial, ISRCTN: 15948785 and T4LIFE trial, NTR3364].

In addition, Nazarpour et al. stratified their analyses based on the TSH concentration at presentation (<4.0 mU/L versus >4.0 mU/L) and found that the benefit of treatment was mainly present in the group with higher TSH concentrations at presentation (preterm delivery risk <4.0 mU/L: 16.7% versus 11.1%, *P*=0.69; for >4.0 mU/L: 29.4% versus 5.3%, *P*<0.01). These findings fit with the synergistically higher risk of adverse outcomes in women with both TPOAb positivity and high TSH as described earlier (see section on subclinical hypothyroidism).[62-65]

FIGURE 2. *The thyroidal response to hCG stimulation in TPOAb negative and TPOAb positive women.*



What remains to be elucidated is the mechanisms through which thyroid autoimmunity is associated with adverse outcomes. We recently showed that the majority of TPOAb positive women have a severely impaired thyroidal response to hCG stimulation (Figure 2).[48] Furthermore, women with high hCG, but lower than expected FT4 concentrations, had a higher risk of premature delivery.[48] However, TPOAb positive women with a combination of hCG and FT4 concentrations similar to TPOAb negative women did not have a higher risk of premature delivery.[48] This indicates that TPOAb positive women lack the hCG-mediated increase in thyroid function during early pregnancy which may lead to a lower thyroid hormone availability (area under the curve) during pregnancy. This may be the mechanism through which thyroid autoimmunity is associated with adverse outcomes. Based on current evidence, TPOAb positive women should be considered as a high-risk group especially when TSH concentrations are high or even high-normal.

Hypothyroxinemia

A normal TSH with low FT4, or hypothyroxinemia, does not fit with the classical perspective of the hypothalamic-pituitary-thyroid axis feedback mechanism. Initially, hypothyroxinemia was considered as a pregnancy-specific disease entity that reflects a state of mild iodine deficiency. However, given that hypothyroxinemia also occurs in iodine sufficient areas and (F)T4 concentrations typically do not increase following iodine supplementation, it is likely that also other physiological processes may lead to hypothyroxinemia.[73-78] Interestingly, in the largest study to date, the subset of women with low urinary iodine concentrations (<100 μg/L or 100-149 μg/L) were not at higher risk of hypothyroxinemia while women with high urinary iodine concentrations (≥500 μg/L) had a 2.9-fold higher risk.[33] This suggests that there is a multifactorial and pregnancy-specific pathophysiology that underlies hypothyroxinemia. This is also in line with the various newly identified risk factors for hypothyroxinemia, such as iron status, placental angiogenic factors, and subject characteristics such as BMI and age.[33,46,52,79,80] In line with the general notion that hypothyroxinemia is a pregnancy-specific disease entity, we have recently shown that the hCG concentration at blood sampling is a determinant of hypothyroxinemia.[12] However, we also demonstrated that the thyroidal response to hCG in women with hypothyroxinemia was similar to euthyroid women.[12] Further studies are required to identify the mechanisms underlying hypothyroxinemia.

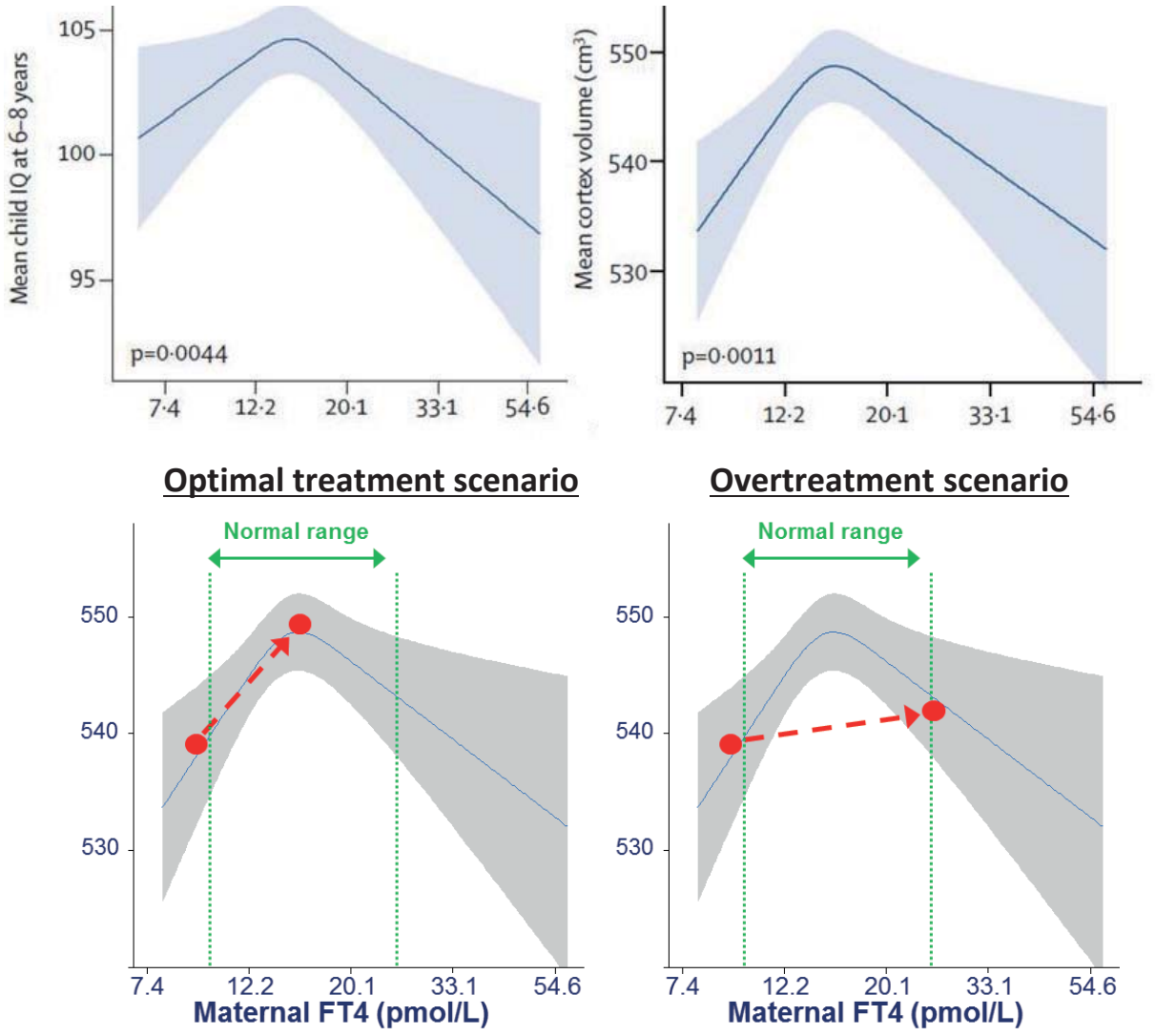
A distinct difference between hypothyroxinemia and subclinical hypothyroidism can be made for the consistency of studies on adverse outcomes. While subclinical hypothyroidism is associated with various adverse pregnancy outcomes, hypothyroxinemia has been predominantly been associated with adverse neurobehavioral outcomes in the offspring.[64,81-83] In 3659 mother-child pairs from a prospective birth cohort, hypothyroxinemia (defined as a TSH <2.5 mU/L with FT4 below the 5th percentile) was associated with a 1.8-fold increased risk of expressive language delay at both 18 and 30 months and a 2-fold increased risk of nonverbal cognitive delay.[84] Follow-up data subsequently revealed that maternal hypothyroxinemia was associated with a 4.3 point lower nonverbal IQ at 6 years of age.[85] When various FT4 cut-offs were assessed, low maternal FT4 up to the 10th percentile was associated with a 1.5-3.8 point lower IQ.[14] Similar findings were also reported by Julvez *et al.* who studied the association of maternal TSH and FT4 (median week 13; range: 8–21) with child mental and psychomotor score at age 14 months in 1643 mother-child pairs in a Spanish prospective birth cohort.[83] They reported a dose-response relationship of low maternal FT4 with child mental score exhibiting a 2.4 point lower child IQ for FT4 below the 10th percentile up to a 3.4 and 4.2 point lower IQ for FT4 levels below the 5th and 2.5th percentile, respectively.[83]

Although IQ is the most studied outcome, adverse effects of hypothyroxinemia have also been demonstrated for other postnatal markers of intrauterine brain development. Maternal

hypothyroxinemia is associated with a 2.6-fold higher risk of clinical autistic symptoms in both boys and girls at a median age of 6 years.[86] Gyllenberg *et al.* showed that maternal hypothyroxinemia (FT4 ≤10th percentile and TSH 5th–95th percentile in early pregnancy) was associated with a 1.7-fold higher risk of schizophrenia (95%CI: 1.13-2.55) by studying 1010 schizophrenia cases matched (1:1) with controls.[87] Hypothyroxinemia is also associated with offspring ADHD, a slower reaction time, suboptimal school performance and lower grey matter and, cortex volume.[14,88-90]

Two randomized controlled trials have assessed the effects of treatment of mild thyroid dysfunction on offspring IQ. In the CATS trial, women were randomized to no screening or screening and subsequent treatment with 150 µg levothyroxine if the TSH concentration was above the 97.5th percentile and/or the FT4 concentration was below the <2.5th percentile.[8] 390 children of treated mothers and 404 children of untreated mothers underwent IQ testing at age 3.[8] There was no difference between the groups in mean IQ or the proportion of children with an IQ below 85 (also not for the low FT4 or high TSH groups separately).[8] Various arguments have been proposed for this negative finding, namely that treatment started too late (median 13 weeks), that IQ cannot be assessed reliably at age 3 or that the loss to follow-up was too high (24%).

FIGURE 3. *The association of maternal FT4 during early pregnancy with child IQ and cortex volume, and postulated treatment scenarios.*



However, recent findings indicate that the relatively high levothyroxine dosage of 150 µg may have contributed to the lack of a net beneficial effect (Figure 3; see also the section on hyperthyroidism).[14,91] linical studies predominantly focused on the effects of low maternal availability on offspring neurodevelopment. However, a more recent study assessing the full range of FT4 concentrations indicated that high FT4 concentrations are associated with suboptimal offspring neurodevelopment similar to the extent of low FT4 concentrations. High maternal FT4 was associated with a 1.4 to 3.7 point lower IQ (N=3839), lower gray matter volume and lower volume of the cortex (N=646).[14] This indicates that high-normal maternal FT4 concentrations may have equally detrimental effects on the fetus and that we can potentially overtreat women that use LT4 during pregnancy. Currently, important work is being carried out by further follow-up of the CATS trial which may further identify late benefits, but also potential harms of maternal levothyroxinetreatment., it may be likely that this trial will be underpowered to show an expected, and relevant, difference in IQ.

The second RCT is a multicenter study from the US, in which almost 100.000 women were screened for thyroid dysfunction.[92] Women with hypothyroxinemia or subclinical hypothyroidism were randomized to receive either a placebo or levothyroxine treatment (50 or 75 µg) and children underwent IQ testing at the age of 3 or 5 years (92.3% assessed at 5 years). When this study was designed (in 2006) the sample size was determined based on the study by Haddow et al. showing a 7 point reduction in offspring IQ of mothers with overt hypothyroidism.[54] The sample size was defined based on the number of randomized women that would allow for detection of a statistically significant difference of 5 IQ points (see trial registration NCT00388297). However, since the start of this trial, prospective cohorts studies have shown that mild maternal thyroid dysfunction is associated with a 3 to 4 IQ point difference.[14,81,83,84] As such, this trial will be underpowered to show the expected difference in offspring IQ and a negative results cannot answer the question whether levothyroxine treatment can reverse the adverse effects of low maternal thyroid function on offspring neurodevelopment. Preliminary results from this trial show that levothyroxine treatment is associated with a median increase of 3 IQ points in the offspring of women with subclinical hypothyroidism and hypothyroxinemia.[92] This IQ increase is seen despite a relatively late start of treatment and is in line with what is expected based on observational data, but due to the inadequate power calculations fails to reach statistical significance.[92] Therefore, further studies are required to elucidate the effects of levothyroxine treatment in women with mild thyroid dysfunction on offspring neurodevelopment.

Studies on the association of hypothyroxinemia with adverse pregnancy outcomes, such as premature delivery or pre-eclampsia, are less consistent.[64,93,94] We therefore speculate that subclinical hypothyroidism, or maternal TSH concentrations in general, mainly reflect maternal thyroid status, whereas thyroid hormone availability for the fetus is predominantly determined by maternal FT4 concentrations independent of TSH, since thyroxine passes the placental barrier (see Figure 4). This is supported by the negative association of maternal FT4 concentrations with for example birth weight and the lack of association for TSH concentrations with offspring outcomes in the majority of studies.[14,82-84,87,89,95-97]

Subclinical hyperthyroidism

Subclinical hyperthyroidism during pregnancy most likely reflects the transient peak in FT4 concentrations that occurs under the influence of hCG.[12] Since subclinical hyperthyroidism mostly has a transient and physiologic nature, there is a lack of association with adverse outcomes, and some studies even indicate a protective effect.[11,64,90,98-100] Alternatively, subclinical hyperthyroidism may represent a subclinical form of Graves' hyperthyroidism, in which case it is likely that the suppressed TSH and high-normal FT4 persist throughout pregnancy. However, persistent high-normal FT4 concentrations may be

unfavorable since studies have shown that higher FT4 concentrations are associated with lower birth weight and lower child neurocognition.[14,82,95] Although women with suppressed TSH but high-normal FT4 may benefit from further follow-up, it is unlikely to be beneficial to lower FT4 concentrations due to the harms associated with antithyroid drugs.

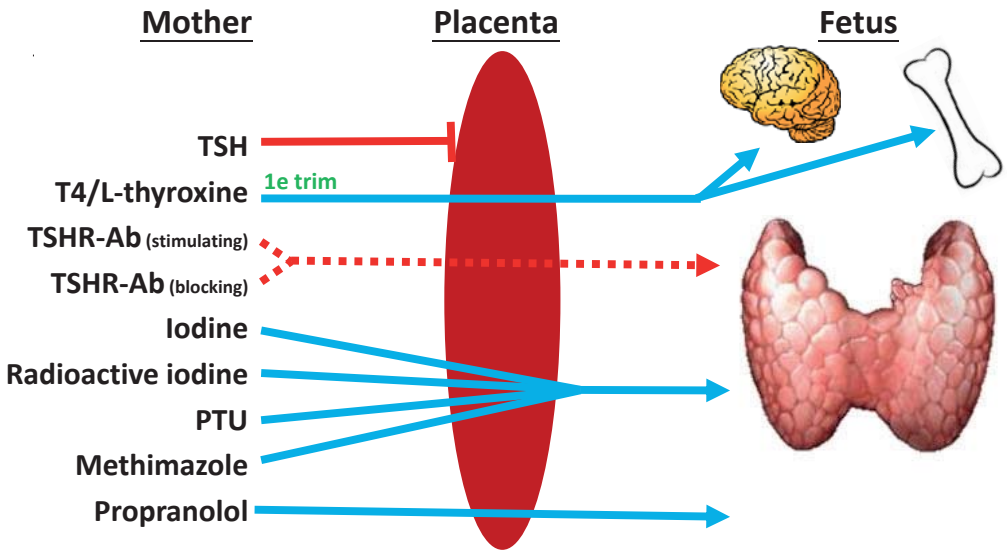
Overt hyperthyroidism

Two major subtypes of overt hyperthyroidism can be distinguished during pregnancy. First of all, there is a pathological form that predominantly consists of women with Graves' disease or autonomous thyroid hormone production (e.g. multinodular toxic goiter or toxic adenoma(s)). This form is rare, with estimated prevalence rates of 0.5-1.3% for pre-existing Graves' disease, 0.05% for new onset Graves' disease, 0.1% for autonomous thyroid hormone production,[13,101] but often presents with clear biochemical abnormalities, symptomatology and is associated with a high risk of adverse outcomes. Secondly, there is a group of women with transiently high thyroid function due to high hCG concentrations that typically peak around the 10th week of pregnancy (Figure 1). Based on population-based reference ranges (i.e. TSH < 2.5th percentile and FT4 >97.5th percentile), this gestational hyperthyroidism occurs in 0.3%-1.0% of all pregnant women.[11,64,102] Some of these women lack hyperthyroidism symptoms, whereas others will classifiy as being transiently thyrotoxic, requiring treatment to relieve symptoms (i.e. beta-blockers).

Overt hyperthyroidism – pathological

Data on the consequences and treatment effects of non-hCG induced hyperthyroidism during pregnancy are sparse and studies predominantly include women with Graves's disease. Consistent associations have been shown with a higher risk of pre-eclampsia, preterm birth, low birth weight and maternal heart failure.[13,103-109] The majority of those studies either lack data on treatment status or only included women that received antithyroid drugs. The transplacental passage properties of various related drugs and thyroid parameters (Figure 4) makes it difficult to identify whether these pregnancy complicationsare caused through maternal hyperthyroidism, fetal hyperthyroidism (caused via the transplacental passage of TRAbs) or the adverse effects of antithyroid drug treatment (including fetal hypothyroidism, risk of anomalies or maternal liver dysfunction).

FIGURE 4. *Thyroid related substances and crossing of the placental barrier.*



A large American record linkage study compared 417 women diagnosed with hyperthyroidism during pregnancy with almost 217,000 controls. Women diagnosed with hyperthyroidism had a 1.8-fold higher risk of pre-eclampsia. In line with a higher risk of pre-eclampsia, women diagnosed with hyperthyroidism also had a 1.2-fold higher risk of threathened preterm birth, a 1.8-fold higher risk of late pretem birth and a 3.7-fold higher risk of maternal ICU admission.[110] A similar study from Denmark showed that women diagnosed with hyperthyroidism have a higher risk of miscarriage, stillbirth, preterm birth, lower birth weight and a child with ADHD.[111-113]

Although the above record linkage studies are prone to include various types of misclassification bias, this is less of an issue for a recent case-control study that investigated 208 hyperthyroid women (89.4% diagnosed before pregnancy, 95% received treatment) and 403 matched controls.[109] Hyperthyroid women had a 3.9-fold higher risk of pre-eclampsia, a 2.2-fold higher risk of fetal growht restriction, a 1.7-fold higher risk of preterm birth and a 3.6-fold increased risk of induction of labor.[109] Interestingly, besides the risk of fetal growth restriction, these risks did not differ between women diagnosed before (N=186) or during pregnancy (N=22).

However, the risk of the previously mentioned adverse outcomes was substantially larger in women that were biochemically uncontrolled (N=45) than in controlled women (N=163). This study, and other observational studies show a benefit of treatment benefit,[106,107] which is in line with studies that did not identify an increased risk of adverse outcomes in women receiving adequate antenatal care.[108] This suggests that the high TH concentrations and not the antithyroid drugs are causing these adverse pregnancy outcomes.[109]

Still, there are potential harms associated with ATD treatment, particularly during early pregnancy. The use of propylthiouracil during pregnancy is associated with maternal liver injury[114] as well as with a higher risk of birth defects such as preauricular sinus/cysts and hydronephrosis.[115,116] The use of methimazole or carbimazole has been associated with cases of agranulocytosis[117,118] and a higher risk of birth defects such as choanal atresia, aplasia cutis and omphalocele.[115,116] To limit the potential adverse outcomes associated with ATD, several pregnancy-specific ATD strategies can be applied. First of all, in women of childbearing age with hyperthyroidism, the option of definitive treament before pregnancy should be discussed. When radioactive iodine is chosen as a definitive treatment, the flare up of TrAb up to one year after treatment should be taken into accout since TrAbs pass the placenta and can stimulate the fetal thyroid. TrAb titers generally tend to remain higher compared to other treatment modalities.[119]

Secondly, if the patient prefers antithyroid drugs or is already pregnant while on thyroid drugs, so-called block and replace therapy is not an option and therapy should consist of monotherapy with the the lowest possible dose. Due to immune modulatory changes during pregnancy, the dose of antithyroid drugs can usually be reduced throughout pregnancy.[120,121] Large population studies have indicated that the use of PTU during early pregnancy is associated with a slightly lower risk of adverse outcomes, but also less severe fetal anomalies, compared to methimazole.[115,116] Therefore, women receiving methimazole who are in need of continuing therapy during pregnancy should be switched to PTU as early as possible and up until the 16th week of pregnancy after which the critical window for fetal organogenesis has passed. Interestingly, a novel study by Yoshiara et al. showed that a novel approach, by which potasium iodide is used to control Graves' hyperthyroidism during the first trimester, may reduce the incidence of congenital anomalies.[122]

Third, in women that are controlled with a low dose of methimazole (5-10 mg) or PTU (100-200 mg) before pregnancy the chance of relapse is lower than the overall 30-50% and relapse would most likely occur after a few months.[123] Therefore, it can be considered to stop the treatment upon the wish to become pregnant or at the time of the first positive pregnancy test (in case of non-planned pregnancy), and monitor thyroid function in order to prevent the negative conseuqences of antithyroid drugs for the fetus.

Finally, an early pregnancy measurement of TrAbs should be performed. If TrAbs are elevated, also the fetus should be monitored from midpregnancy onwards via for example fetal heart rate, fetal growth and/or fetal thyroid ultrasound. Together with the maternal thyroid function, these measures are a reflection of the fetal thyroid hormone status.[124,125]

Overt hyperthyroidism – gestational

Gestational hyperthyroidism is mostly considered a non-pathological entity because it is driven by the physiological peak in hCG. Despite the fact that gestational hyperthyroidism generally reflects normal physiology, some[82,95,98,126] but not all[106,127-131] studies on the association of gestational hyperthyroidism have shown a higher risk of lower birth weight and a higher risk of pre-eclampsia. However, these studies lacked measurement of TrAbs and may therefore have been subdue to misclassification (i.e. classification of Graves' disease as gestational hyperthyroidism). To circumvent this misclassification, we recently studied the combination of thyroid function with hCG and showed that women with high hCG and high thyroid function do not have a higher risk of pre-eclampsia while women high thyroid function despite with low hCG (presumable due to TrAbs or autonomous thyroid hormone secretion) have an up to 11-fold higher risk of pre-eclampsia.[126] This indicates that an additional hCG measurement may help to distinguish physiological from pathological forms of hyperthyroidism during pregnancy. These data warrant further studies on the different subtypes of biochemical hyperthyroidism.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recently published studies have added to our interpretation of the definition of a normal or abnormal thyroid function during pregnancy. It is still evident that the most specific way to define reference ranges is a population-based approach. Fortunately, the wide range of available studies now also allow for adoption of such reference ranges or a more evidence-based recommendation on a fixed cut-off. However, recent studies indicate that risk of adverse outcomes according to thyroid function is a continuous spectrum, as opposed to binary (i.e. defined only through reference ranges). Furthermore, the risk of adverse outcomes also seems to be conditional on thyroid function determinants (i.e. TPOAb positivity, hCG) and to differ according to combinations of thyroid function associated measurements (i.e. TPOAbs and TSH concentrations). Further elucidation of such conditional risks and threshold will allow us to distinguish high-risk individuals that may potentially benefit from treatment, and this may also help to overcome unnecessary treatment. Future studies are needed to identify more specific, percentile-based, disease risk thresholds and collaborative efforts are currently set-up to study this.[132] Further studies on the potential of thyroid hormone determinants such as thyroid autoimmunity, hCG, endocrine disrupting chemicals and iodine may also prove valuable in improving our clinical interpretation of at risk individuals. The field currently lacks clinical trial data, and although these are necessary to prove that there are potential benefits of treatment, the potential risks associated with overtreatment associated with levothyroxine treatment during pregnancy requires further studies and caution.

SUPPLEMENTAL TABLE 1. *Reference ranges for TSH and FT4 during early pregnancy worldwide.*

Author, Country (reference) (analyzing method)	N	Gestation (week)	TSH in mU/L		FT4 in pmol/L (ng/dl)			Population characteristics		
			Median	2.5th-97.5th	Median	2.5th-97.5th	(Median, 2.5th-97.5th)	Iodine deficiency	Mean BMI	Ethnicities (%)
Bestwick *et al.*, Italy (21) (AutoDELFIA)	5505	<16	1.07	0.04 - 3.19	9.3	7.4 - 12.2	(0.73, 0.58 - 0.95)	Moderate-Mild	[a]	NR
Bestwick *et al.*, UK (21) (Advia Centaur)	16,334	<16	1.11	0.06 - 3.50	13.9	10.9 - 17.9	(1.08, 0.85 - 1.40)	Moderate-Mild	[a]	NR
Bliddal *et al.*, Denmark (Modular E170)	455	T1		0.1 - 3.6		11.7 – 19.1	(, 0.91 – 1.49)	Sufficient	22.7	Caucasian (predominantly), Afrocaribian, Asian, Oriental.
Bocos-Terraz *et al.*, Spain (9) (Architect)	481	<14	0.94	0.41- 2.63	13.9	10.8 – 17.8	(1.08, 0.84 - 1.38)	Mild	NR	White (93%)
Gilbert *et al.*, Australia (22)[b] (Architect)	1817	9-13	0.74	0.02 - 2.15	13.5	10.4 - 17.8	(1.05, 0.81 - 1.39)	Borderline	NR	Australian
Lambert-Messerlian *et al.*, USA (23)[c] (Immulite 2000)	8351	T1	1.00	0.12 - 3.37	14.2	10.4 – 17.8	(1.10, 0.81 - 1.38)	Mild	NR	White (67) and Hispanic (23)[d]
	8415	T2	1.19	0.35 - 3.35	13.0	9.3 – 16.2	(1.01, 0.72 - 1.26)			
La'ulu *et al.*, USA (24,25)[e]	2172	10-13	0.94	0.02 - 2.69	14.7	11.4 - 18.6	(1.15, 0.89 - 1.45)	Mild	NR	Hispanic (37), White (29), Black (27), Asian (8)
	2683	14-20	1.14	0.15 - 3.11	12.0	9.3 - 15.2	(0.94, 0.73 - 1.19)			
Li *et al.*, China (26) (Cobas Elesys 601)	640	7-12	1.47	0.10 - 4.34	15.8	12.3 - 20.9	(1.23, 0.96 - 1.63)	Proven sufficient[f]	NR	Chinese (presumed)
Männisto *et al.*, Finland (27) (Architect i2000)	4333	T1	1.11	0.08 – 3.54	15.3	11.7 – 22.8	(1.12, 0.86 - 1.58)	Sufficient	22.4	Finnish (presumed)
	747	T2	1.37	0.11 – 4.24	14.6	11.2 – 23.4	(1.13, 0.87 – 1.82)			
Medici *et al.*, the Netherlands (28) (Vitros ECI)	5186	8-18	1.30	0.03 - 4.04	14.7	10.4 - 22.0	(1.15, 0.81 - 1.72)	Proven sufficient[f]	24.5	Dutch (52), Surinamese/Antillean (12), Turkish (8), Moroccan (6)
Mosso *et al.*, Chile (Modular E 170)	647	<15	1.96	0.11 – 5.96	14.5	11.1 – 19.0	(1.13, 0.87 – 1.48)	Sufficient to excessive	26.3	Presumed Chilean
Pearce *et al.*, USA (29) (Advia Centaur)	585	<14	1.1	0.04 - 3.60	2.1[h]	1.5 - 2.9[g]	-	Borderline	NR	White (77) and Black (10)
Quinn *et al.*, Russia (30) (Abbott AxSYM)	380	T1	1.66	0.09- 4.67	-	-	-	Moderate	NR	Russian (presumed)
	549	T2	2.00	0.20- 4.68	-	-	-			
Springer *et al.*, Czech Republic (31)[h] (ADVIA Centaur)	4337	9-11	1.21	0.06 - 3.67	-	-	-	Mild	NR	Caucasian (99)
Stricker *et al.*, Switserland (14) (Architect i2000SR)	575	6-12	0.95	0.07 - 2.82	13.9	10.5 - 18.5	(1.08, 0.82 - 1.44)	Sufficient	NR	Swiss (presumed)
	528	T2	1.02	0.20 - 2.79	12.2	9.5 - 15.7	(0.95, 0.74 - 1.22)			
Vaidya *et al.*, UK (32) (Modular E 170)	1089	<12	1.08	0.14 - 3.19	14.6	10.7 - 19.4	(1.12, 0.83 - 1.59)	Mild-moderate	NR	White (91) and South Asian (4)
Suleyman *et al.* Turkey (Architect i2000SR)	945	T1	1.29	0.49 – 2.33	13.4	10.3 – 18.1	(1.05, 0.80 – 1.41)	Moderate	NR	Middle East, Balkans, Eastern Europe/Caucasian.
	1120	T2	1.58	0.51 – 3.44	13.3	10.3 – 18.2	(1.04, 0.80 – 1.42)			
Xing *et al.* China (Henan) (Immulite 2000)	398	T1	1.30	0.07 – 3.96	13.1	9.2 – 18.1	(1.20, 0.72 – 1.41)	Sufficient to excessive[j]	NR	Chinese
	797	T2	1.64	0.27 – 4.53	11.7	7.8 – 13.9	(0.91, 0.61 – 1.09)			
Zhang et al., China (Nanshan) (Beckman Coultier, DxI 600 Access)	1521	T1		0.06 – 3.13		8.7 – 15.2	(, 0.68 – 1.19)	Sufficient	[i]	Chinese (presumed)
	1102	T2		0.07 – 4.13		7.1 – 13.6	(, 0.55 – 1.06)			

Studies were selected according to the following criteria: N≥500, exclusion of TPOAb positive women and availability of data from the manuscript or via personal communication. Iodine status was estimated based on references from article, WHO iodine status reports or from the Vitamin and Mineral Nutrition Information System (VMNIS).
TSH, thyroid-stimulating hormone; FT4, free thyroxine; NR, Not reported; T1, first trimester; T2, second trimester.
[a] Weight reported (Bestwick et al. median weight 59 kg in Italian and 67 kg in UK population); [b] Reported FT4 level is a mean; [c] Limits are 5th and 98th percentiles for TSH and 2nd and 95th percentiles for FT4; [d] Based on reports of the total FASTER population; [e] FT4 determined in normal-range TSH only; [f] Based on iodine measurements in study population; [g] Free T4 index (normal range 1.0-4.0); [h] High hCG levels excluded; [i] median weight 1st trimester 52kg (41–70), second trimester 52kg (41–72); [j] Study selected healthy women, consuming iodized salt only.

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CHAPTER 19

CLINICAL ASSOCIATIONS OF MATERNAL THYROID FUNCTION WITH FETAL BRAIN DEVELOPMENT: INTERPRETATION AND OVERVIEW OF AVAILABLE EPIDEMIOLOGICAL EVIDENCE

Tim I.M. Korevaar, Henning Tiemeier and Robin P. Peeters

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Thyroid hormone is an important regulator of metabolism, growth and development in practically all tissues in the human body. During pregnancy, major changes occur in thyroid physiology including an increase in thyroid hormone binding globulin, an increase in thyroid hormone degradation by thyroid degrading enzymes (due to placental expression of deiodinase type 3) and an increase in thyroid stimulation by human chorionic gonadotropin (hCG). Overt maternal thyroid disease, mainly overt hypothyroidism and Graves' hyperthyroidism, occurs in approximately 0.5% of all women and is a well-known modifiable risk factor for a wide range of adverse pregnancy and child outcomes.[1] However, more recent studies have shown that also milder forms of maternal thyroid dysfunction, particularly subclinical hypothyroidism and hypothyroxinemia, are associated with a higher risk of similar adverse outcomes such as pre-eclampsia, premature delivery, abnormal birth weight and impaired offspring neurobehavioral development. Although the risk of adverse outcomes is not nearly as high as compared to overt disease, the combined prevalence of subclinical thyroid disease entities is at least 8%, which makes it an important public health issue.

In the field of thyroid and pregnancy, the hypothesis that the placental transfer of maternal thyroid hormones to the fetus is associated with fetal brain development has gained particular interest. This is mainly because of the specific, time-dependent, underlying biology and the major public health effects of suboptimal neurocognitive ability. This review aims to outline this specific hypothesis and the caveats that come with testing it in a clinical setting. It will also comprehensively summarize and connect the results of all recent clinical studies that have focused on different clinical neurobehavioral outcomes and discuss future perspectives.

Interestingly already more than 100 years a-go, the link between maternal goiter during pregnancy and cretinism of the offspring was described.[2] Given the marked intellectual impairment of cretins, the link between maternal thyroid hormones and child brain development has been known for long yet it was not until the 1950s that further studies assessed this association.[3-5] Seminal studies in animals showed that hypothyroid rat pups had differently shaped and also smaller brains, with smaller pyramidal cell bodies in the cerebral cortex. Subsequent studies made it apparent that thyroid hormone regulates migration, proliferation, and differentiation of fetal neuronal cells, as well as synaptogenesis and myelination.[6,7] The study epidemiological study was performed by Man et al. in 1969, the authors screened 1394 pregnant women for thyroid deficiency, defined as the combination of low butanol extractable iodine and inadequate thyroid replacement therapy.[8] They reported that women with gestational thyroid deficiency had 8-month-old infants of which only 48% were classified as 'normal' on psychological testing compared to 73.4% for infants of mothers with the usual gestational increase in serum thyroxine-like iodine.[8]

Subsequent studies have taught us that, in humans, various thyroid hormone dependent early brain development processes, such as neuronal proliferation, migration and differentiation, commence in the 5th week of pregnancy. Fetal thyroid hormone receptors are present from at least the 8th to 10th week of pregnancy yet the fetal thyroid itself is not functionally matured until week 18-20.[9] As such, early fetal brain development is dependent on the placental transfer of maternal thyroid hormones. In a clinical setting, it is difficult to test the hypothesis that maternal thyroid hormones, as transported over the placental barrier, regulate fetal brain development. There are three major factors that complicate human/epidemiological studies:

(I) First of all, it is simply not possible to perform prospective, in vivo studies in humans. Therefore, we can only study the association between proxy measures of the physiological processes of interest, obtained through minimally invasive procedures. For the exposure, we approximate the placental thyroid hormone transfer (or more specifically the maternal thyroid hormones that reach the fetal brain) by measuring the maternal thyroid function in serum. For the outcome, we approximate inter-individual

differences in intrauterine fetal brain development during the postnatal period by neurocognitive tests, the occurrence of brain disease or brain imaging modalities. Because there is no other way to assess the intrauterine differences in brain development, we have to accept that its approximation comes at the price of making assumptions on the biological plausibility and, measurement error. For example, we have to assume that a maternal serum thyroid function measurement (e.g. TSH and FT4 concentrations) is a proper proxy for placental thyroid hormone transfer during early pregnancy. Also, we have to accept that TSH and FT4 measurements come with instrumental and physiological variation and the same goes for intelligence quotient (IQ) testing. The combination of biological (e.g. gestational thyroid hormone fluctuations) and methodological (e.g. assay reproducibility) measurement variability leads to a random error (as opposed to systematic error when all variables are for example +10%) that is non-differential (e.g. not associated with the outcome variable). In other words, it is unlikely that in a specific group of individuals the same error affects each measurement of thyroid function (making it random) and it is especially not likely that this error will affect mothers of children that will later have a lower IQ during later life (making it non-differential). Random measurement error of a continuous exposure variable (i.e. FT4 concentrations) attenuates effect estimates in clinical association studies, the extent of which is associated with the extent of measurement error. This attenuation not only inadequately reflects the true effect, the decrease in the effect size makes it more difficult to show that a finding does not occur due to chance (i.e. it decreases statistical power). This attenuation effect can be overcome by either using better proxies (like equilibrium dialysis or repeated measurements) or by performing studies that are large enough and have adequate statistical power to detect smaller effects.

(II) we also need to take into account how our study methodology can be influenced. (A) Changes in the study population may occur after inclusion, this may lead to follow-up bias, which can for example occur when women with low thyroid function are less likely to participate in the second phase of the study when neurocognition is assessed. Such a bias was recently seen in a Dutch study that investigated studied school performance. This problem was, to an extent, coped with by using inverted probability weighting (which inflates the weight for subjects without follow-up data), showing a reduction of the differences in the outcome.[10] (B) Unmeasured variables can cause residual confounding, which is a limitation of observational studies making it impossible to exclude that the identified association is still confounded by a factor that was not measured (and for which you thus cannot statistically adjust). (C) Biological variation, for example through differences in the downstream effects of thyroid hormone or brain developmental plasticity, can lead to inter individual differences in the effect. In other words, for individuals with the same amount of thyroid hormone exposure there is a different effect on neurodevelopment. This unmeasured effect modifier may also interfere with identifying the true effect. (D) Effect modification could also occur via postnatal child development differences. It could be possible that the effect of maternal thyroid function is actually stronger when the postnatal child thyroid function is low instead of high. Furthermore, postnatal differences in child development could potentially mediate the effect of maternal thyroid function on child neurodevelopment. Effect mediation can for example occur when maternal thyroid function leads to alterations in child thyroid function, and differences in child thyroid function subsequently affect postnatal brain development. Although this does not necessarily change the association of interest, identification of such mechanisms may prove valuable when findings want to be extrapolated to the clinic or an interventional study. In our previous study we were able to show that the association of maternal thyroid function with child IQ and brain morphology was not mediated or modified by differences in postnatal child thyroid function.[11]

(III) Finally, from a more pragmatic point of view, it is also difficult to study the discussed hypothesis due to the need for extensive resources. Thyroid dysfunction during pregnancy has a relatively low prevalence and the described methodological consideration requires access to a large group of pregnant

women for whom either early pregnancy serum or a thyroid function measurement is available. Subsequently, follow-up is needed of the mother-child pairs for at least a few years in order to obtain a neurodevelopmental test outcome for the child. This often requires an extensive infrastructure that is costly of time, money and collaborative efforts.

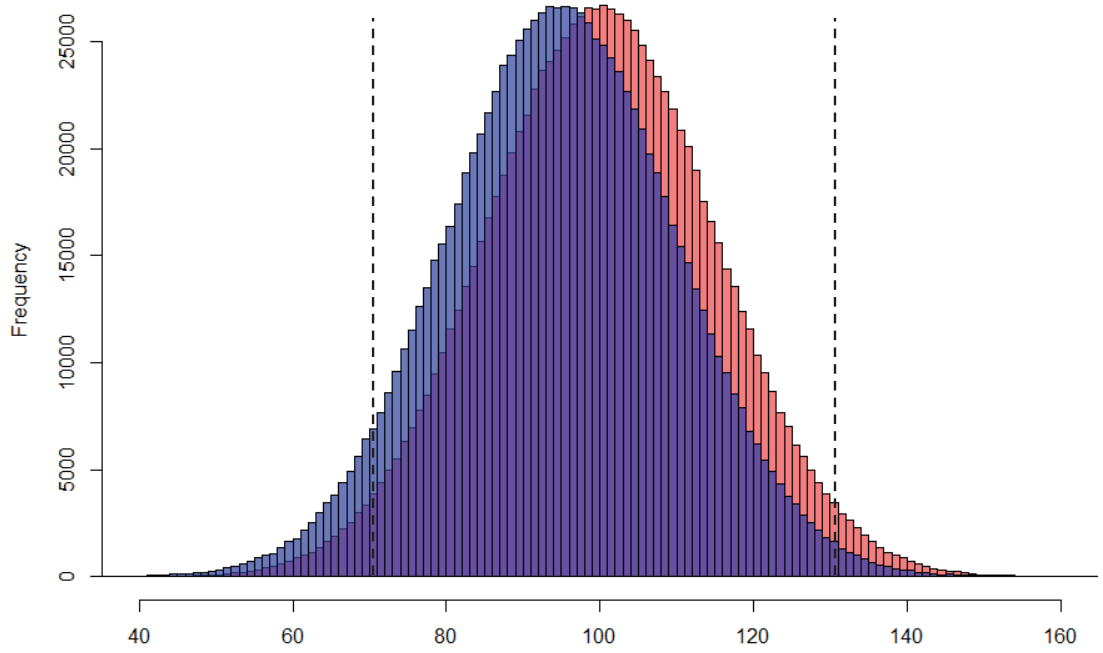
Despite all of these epidemiological considerations, various large studies have been performed while some circumvented some of the issues described above (for example by performing case-control studies). Clinical studies so far can be divided into observational studies and randomized controlled trials (RCTs) that investigate the potential benefit of levothyroxine supplementation. Observational studies can be further subdivided according to the endpoints that were studied (IQ, brain disease, brain morphology and school performance outcomes) and this usually explains differences in the study design (cohort or case-control).

Offspring IQ

To date, IQ is the best studied neurobehavioral outcome to test the hypothesis that maternal thyroid hormones regulate fetal brain development. IQ test outcomes are a standardized outcome with a population mean of approximately 100 and a standard deviation of 15. An IQ assessment is often the assessment of choice as it is one of the easiest and cheapest way to assess general neurocognitive ability in a large group of people. Although the effects of the prenatal environment on child IQ have long been assumed to be negligible, it has been estimated that the prenatal environment accounts for 20% of the variation in IQ.[12] In itself, IQ is a powerful outcome as is reflected by studies showing that childhood IQ scores are consistently associated with adverse outcomes during later life and lifetime achievements including learning abilities, educational attainment, quality of life, and adult health such as cardiovascular disease and mortality.[13,14]

The mean IQ of a population is a stable figure and the variation is large (with a 95% range between approximately 70 and 130), as such it is unlikely to identify large differences in mean IQ scores in a clinical study. Mean IQ as a study outcome is often misinterpreted and unrightfully critiqued as differences in the mean IQ of 3 to 5 points are deemed irrelevant. However, a 5 point differences in IQ for an individual could mean the differences between school levels. Furthermore, from a population perspective, a 5 point IQ difference has many different implications. First of all, already small changes in mean IQ of a population have major consequences for the proportion of individuals that are intellectually disabled (IQ<70), have learning disabilities (IQ<85) and also the proportion of 'gifted' individuals (IQ>130). These effects are shown in Figure 1, which shows the distribution curves of population IQ according to different means and the number/proportion of individuals with IQ below certain thresholds (based on a modelling exercise by TK based on ref[11]). In this population of 100 million individuals in which 5% of individuals have been exposed to low, and 5% to high maternal thyroid hormone concentrations during early pregnancy, and this is associated with an average 3 point lower mean IQ (3.55 and 3.12 points lower, respectively). A 3 point lower mean IQ in the affected 10% of the population would translate into an additional 130.634 individuals with an intellectual disability (+5.7% in the whole population) and an additional 528.714 individuals with a learning disability (+3.1% in the whole population). On the other hand, there would be 88.378 gifted individuals less (-3.7% in the whole population). The evidence for adequate treatment modalities that are able to reverse the changes seen in mean IQ will be discussed further on in this review.

FIGURE 1. *The population effects of a shift in mean IQ.*



	Effects on intellectual disability						Effects on learning disabilities						Effects on giftedness					
	Whole population				Low/high FT4		Whole population				Low/high FT4		Whole population				Low/high FT4	
Mean IQ	**N <70**		**% <70**		**% <70**		**N <85**		**% <85**		**% <85**		**N >130**		**% >130**		**% >130**	
100	228.251	*ref*	2,28	*ref*	2,28	*ref*	1.588.604	*ref*	15,9	*ref*	15,90	*ref*	227.528	*ref*	2,27	*ref*	2,27	*ref*
99	265.378	37.127	2,66	16,3	2,32	1,6	1.752.500	163.896	17,5	10,3	16,04	0,9	194.142	-33.386	1,94	-14,7	2,24	-1,3
98	309.382	81.131	3,08	35,5	2,36	3,4	1.930.053	341.449	19,3	21,5	16,21	2,0	164.384	-63.144	1,64	-27,8	2,21	-2,7
97	358.885	130.634	3,59	57,2	2,41	5,7	2.117.318	528.714	21,2	33,3	16,40	3,1	139.150	-88.378	1,39	-38,8	2,19	-3,7
96	415.289	187.038	4,16	81,9	2,47	8,2	2.316.280	727.676	23,2	45,8	16,60	4,4	117.250	-110.278	1,18	-48,5	2,16	-4,7
95	478.838	250.587	4,78	109,8	2,53	10,9	2.526.428	937.824	25,3	59,0	16,81	5,7	98.213	-129.315	0,98	-56,8	2,14	-5,6

** Calculated based on a population of 100 million in which 10% is expected to have a mean difference in IQ due to suboptimal maternal thyroid function in pregnancy (based on Korevaar et al 2016; 5% low FT4 and 5% high FT4).*

Lower (childhood) IQ has been associated with a higher risk of obesity, high blood pressure, poor educational attainment, memory decline, adverse emotional development, psychological problems, cardiovascular disease, cancer, and premature mortality.[13,14] Furthermore, it has been estimated for that a mean loss of 5 points would cost $275 billion to $326 billion per year in the United States.[15] In addition, exposure to common endocrine disrupting chemicals, that have been associated with similar IQ losses as suboptimal maternal thyroid hormone, has been estimated to costs more than €150 billion per year in the European Union, and even more in the USA.[16,17]

The first study using IQ as an outcome was performed in 1971, Man and colleagues showed that inadequately treated hypothyroxinemic pregnant, as identified using butanol-extractable iodine (a measure of protein-bound or serum precipitable iodine as a marker of thyroid hormone and/or function[18,19]), had children with a lower IQ at age 4 and age 7.[20,21] Some of the first subsequent studies on child IQ were performed by Pop et al. in 1995 and 1999, with a follow-up study published in 2003.[22-24] In a study population of 220 mother and child pairs, the authors showed that mothers with a FT4 below the 10th percentile during the 12th week of pregnancy had children with lower psychomotor development scores (uncorrected difference: -7.4 points; 95% CI: 1.1-13.9) as compared to the offspring from women above the 10th percentile. There was no association with mental development scores or when low FT4 was measured during week 32. Subsequently, Haddow et al. performed a case-control study in which women with overt hypothyroidism (47/62 women had a TSH > 99.7th percentile; 15/62 women had TSH between the 98th and 99.6th percentiles with serum T4 concentrations <7.75 µg/dL, or <99.7 nmol/L) that were either treated or untreated, were compared to euthyroid women.[25] The authors demonstrated that women with overt hypothyroidism around week 17 had children with a consistent pattern of lower intelligence, attention, language development, reading ability and visual-motor performance scores as compared to women with a normal TSH. Not all of the analyses on these different outcomes reached statistical significance (range *P*=0.01-0.40). However, the differences became much more apparent after excluding women (n=14) that were treated during pregnancy and had children with normal IQ levels.[25] They were then able to show that children from mothers with untreated gestational overt hypothyroidism had a 7 point lower IQ compared to children from women in the control group.[25] These studies were the first to translate the phenotype of cretinism and findings from animal studies into clinical studies, supporting the hypothesis that maternal thyroid hormone availability affects fetal brain development.

Subsequently, a study amongst 500 women within a prospective birth cohort investigated the association of maternal TSH, total T4 and TPOAbs assessed at a mean of 10 weeks with child neurocognition at 6 months and 3 years of age.[26] There was no association of maternal TSH, total T4 or TPOAbs with child neurocognition. Given the previous studies, these negative results were contrary to what was expected and the authors speculated that they lacked statistical power. Interestingly, we have recently replicated that maternal total T4 concentrations during early pregnancy are not associated with child IQ.[27] Subsequently, Henrichs et al. studied the association of maternal TSH and FT4 with the risk of expressive language delay at age 18 and 30 months and nonverbal cognitive delay at age 30 months in 3659 mother-child pairs from a prospective birth cohort.[28] They found that hypothyroxinemia (defined as a TSH <2.5 mU/L with FT4 below the 5th percentile) was associated with a 1.8-fold increased risk of expressive language delay at both 18 and 30 months and a 2-fold increased risk of nonverbal cognitive delay. Similar findings were subsequently reported by Julvez et al. who studied the association of maternal TSH and FT4 (median week 13; range: 8–21) with child mental and psychomotor score at age 14 months in 1643 mother-child pairs in a Spanish prospective birth cohort.[29] The results of their study showed a dose-response relationship of low maternal FT4 with child mental score exhibiting a 2.4 point lower child IQ for FT4 below the 10th percentile, up to a 3.4 and 4.2 point lower IQ for FT4 levels

below the 5th and 2.5th percentile, respectively. In line with these results, a recent study demonstrated that hypothyroxinemia (defined as TSH <2.5 mU/L and FT4 below the 5th percentile) was associated with a 4.3 point lower nonverbal offspring IQ as assessed at age 6.[30]

Although studies on the association of maternal thyroid function during early pregnancy with child IQ have predominantly focused on a shortage of thyroid hormones, we recently investigated this association over the full range of maternal thyroid function. In line with various animal studies,[31-35] we identified an inverted U-shaped association of maternal FT4 with child IQ at age 6, suggesting equally adverse effects of high maternal FT4 as low maternal FT4.[27] We assessed various cut-offs and found that low maternal FT4 up to the 10th percentile were associated with a 1.5-3.8 point IQ reduction and high maternal FT4 down to the 88th percentile were associated with a 1.4-3.7 point reduction.[27] These results were independent of differences in maternal hCG or child thyroid function.

Taken together, the various cut-offs for low and FT4 concentrations and dose response relationships that have been reported may suggest that there is a continuous spectrum of the effects of maternal FT4 on child IQ with an optimal concentration of FT4. Although TSH has always been considered as the best reflection of thyroid function during pregnancy, practically all studies that assess child neurodevelopment outcomes find an association with maternal FT4 but not TSH. This is in line with the physiology that maternal thyroid hormones, but not TSH, pass the placenta. In turn, subclinical hypothyroidism is predominantly associated with obstetrical outcomes including miscarriage, preterm birth and breech presentation at birth. Therefore, we postulate that TSH is a better reflection of the maternal thyroid state while FT4 is more likely to represent the amount of thyroid hormone that is available for the fetus, specifically before the fetal thyroid function is functionally matured at week 18-20.[9]

Brain disease entities

Neurobehavioral disease entities such as autism spectrum disorder (ASD), ADHD and schizophrenia are associated with obvious and extensive personal disabilities and public health consequences. Twin studies show a large shared genetic overlap for ASD, ADHD and intellectual disability.[36-39] Although a large proportion of the variability in neurobehavioral disease has been estimated to be genetic, the majority of genetic risk factors remain to be identified.[40-42] This indicates an important role for environmental and developmental risk factors in the underlying pathophysiology. Because brain disease entities may be a more overt representation of disruptions of early neurogenesis they could be a very interesting outcome to study the currently discussed hypothesis. On the other hand, their incidence is low and the diagnosis can be dependent on the physicians interpretation. Therefore, the use of a case-control study design is often necessary and a binary disease outcome is commonly ascertained using standardized diagnosis criteria or using a standardized, continuous symptom scale.

The incidence of ASD is rising and its underlying pathophysiology is considered to be of developmental origin.[43] More specifically, ASD has been connected with aberrant neuronal migration, a process regulated by thyroid hormone.[44] In over 4000 mother and child pairs, Roman et al. demonstrated that maternal hypothyroxinemia (TSH <2.5mU/L and FT4 <5th percentile) was associated with an up to 3.9-fold higher risk of autism and higher overall ASD symptoms scores.[45] A Danish registry-linkage study in a very large study population identified women with thyroid disease in the perinatal period by hospital diagnosis and/or thyroid medication prescriptions. They found that maternal hypothyroidism was associated with a higher risk of ASD (HR 1.30 [1.11-1.53]) while maternal hyperthyroidism was associated with a higher risk of ADHD (HR 1.18 [1.03-1.36]). Although both of these associations were only present if the diagnosis was made after the birth of the child, this could suggest that women were untreated during pregnancy, or only developed thyroid dysfunction after pregnancy, for example in the case of postpartum thyroiditis which may suggest a role for thyroid autoimmunity.

The literature on the association of maternal thyroid function with offspring ADHD is less consistent. In Dutch studies, maternal hypothyroxinemia and TPOAb positivity was associated with higher ADHD symptomatology.[46,47] An Italian study also identified that hypothyroxinemia was associated with a higher risk of ADHD, while a large Finnish study only found an association of maternal TSH, but not low FT4 or TPOAb positivity, with offspring ADHD scores.[48,49]

Schizophrenia is a severe, debilitating disorder that has various neurodevelopmental risk factors and is associated with lower neurocognitive abilities.[50,51] Schizophrenia has a low incidence of 11 to 70 cases per 100.000 individuals and this makes it a very difficult endpoint to study, practically necessitating a case-control study design.[50] Gyllenberg et al. showed that maternal hypothyroxinemia (FT4 ≤10th percentile and TSH 5th–95th percentile in early pregnancy) was associated with a 1.7-fold higher risk of schizophrenia (95%CI: 1.13-2.55) by studying 1010 schizophrenia cases matched (1:1) with controls.[52] Intriguingly, opposite to their pre-specified hypothesis, the authors also identified that women with subclinical hyperthyroidism had a 1.9-fold higher risk of schizophrenia (95%CI: 1.14-3.20), which may again implicate that both too low and too high maternal thyroid function leads to adverse offspring neurodevelopment.[52]

School performance

School performance is an interesting outcome as it can be considered as to be a more 'real-life' outcome than IQ. Interestingly, IQ is highly correlated with educational attainment (typically ranging from r=0.5-0.7)[53] and it has been estimated that every year of schooling enhances IQ by 0.3-0.6 points.[54] The pitfall of school performance as an outcome is the fact that it is usually comprised of a single test that examines knowledge and skills taught over a long period of time. During a long period of time, characteristics including perseverance and homework discipline influence the outcome and for example life-events or other factors that influence learning possibilities or performance can occur. School performance as a study outcome may therefore incorporate more (random) measurement error than an IQ test taken in a research setting.

Relatively small recent studies on school performance have shown non-consistent results. In a subset of the ABCD cohort (N=1196), Noten et al. found that maternal hypothyroxinemia was associated with a 1.5 to 1.6-fold higher risk of suboptimal school performance on an arithmetic test, but not for a language test, at the age of 5 years.[55] Notably, they did not find any associations for maternal subclinical hypothyroidism or continuous analyses of TSH or FT4 concentrations and the results for hypothyroxinemia attenuated slightly after inverse probability weighting. A similar study by Päkkilä et al. assessed the association of maternal thyroid dysfunction with teacher reported difficulties in reading/writing and mathematics at age 7 to 8 years (N=5069/5078), with self-evaluated school performance for language and mathematics at age 16 years (N=4357/4370) and with child intellectual problems (defined through record linkage; N=5791).[56] This study did not show any consistent association of maternal hypothyroidism, hypothyroxinemia or hyperthyroidism with one of the outcomes.[56] As children from mothers that participated in birth cohorts during pregnancy are getting older, more studies will be able to evaluate the effects of maternal thyroid function on school performance of the offspring. However, the high measurement error of the outcome as well as the clustering of children into classes and schools require large numbers and advances statistical techniques to correctly identify potential effects.

Maternal thyroid function is strongly associated with neonatal or childhood thyroid function presumably via a combination of fetal hypothalamic-pituitary-thyroid axis set point adaptivity to maternal thyroid function, direct placental transfer of maternally produced thyroxine and genetic overlap.[57] As such, neonatal thyroid function is likely to, partially, reflect maternal thyroid hormone status. Recently, Lain et al. were able to study the association of neonatal TSH measurement, performed

during heelprick screening in Australia, and educational results in over half-a-million children.[58] The authors demonstrated that neonatal TSH concentrations from the 90th percentile upwards were associated with an up to 75% and 42% higher risk of a score below national minimum standard for numeracy and reading, respectively. Similarly, neonatal TSH concentrations from the 98th percentile upwards were associated with a higher risk of vulnerability in developmental domains or having special needs.[58] The main limitation of this study is that data was ascertained through record linkage, for which misclassification bias and residual confounding are important limitations. Nonetheless, the results suggest that fetal thyroid hormone availability is associated with school performance outcomes and this study exhibited adequate statistical power to analyze thyroid function as a continuous exposure and detect differences in school performance outcomes.

Brain morphology

The objectiveness and lack of day-to-day variation of offspring brain morphology as assessed by magnetic resonance imaging (MRI) likely makes it a good proxy for differences in fetal brain development. Notably, neuroimaging outcomes are associated with other outcomes such as IQ and brain disease.[59-63] The downside of using neuroimaging outcomes as a proxy for differences in fetal brain development include the high financial costs and larger effort required to be put in by both the study subjects and the researcher staff.

The first studies on maternal thyroid and offspring neuroimaging outcomes were performed by the group of Rovet et al. They composed a study population consisting of 20 to 24 children (~30% of an original birth cohort) born from mothers that were undertreated for overt hypothyroidism (HYPO group) during at least a part of pregnancy, and 20 to 30 healthy controls. The group of children in the HYPO group had smaller hippocampi, differently sized sub regions of the corpus callosum and differential, but notably, only unilateral thinning or thickening of certain cortical regions at the age of 10 to 12 years.[64-66] In addition, interesting hints towards dose-dependent effects and differences according to thyroid dysfunction severity were identified.[64-66] Notably, replication of these findings will be important as the small sample size of these studies combined with the mild multiple testing corrections and lack of data on important confounders gives a high risk of false positive findings.

A subsequent study, performed using the Generation R cohort, studied the association of maternal thyroid function with offspring MRI outcomes available in 652 mother-child pairs. In line with previous studies, cut-offs for low thyroid function and linear associations were studied but no differences were identified for brain volumetric measures, cortical thickness, and surface area between children exposed prenatally to hypothyroxinemia and controls.[30] Re-analysis identified that there was an inverted U-shaped association of maternal FT4 concentrations with gray matter volume and also cortex volume (e.g. gray matter not from subcortical regions). These data further strengthen the findings that also high maternal thyroid hormone concentrations may have adverse effects on offspring neurodevelopment. Given the specificity and precision of brain imaging, further studies using brain imaging modalities can prove valuable in unravelling the effects of prenatal thyroid hormone exposure and fetal brain development.

The effects of treatment

Randomized intervention studies are the key to unravelling the causality suggested by observational studies and to investigate whether maternal thyroid hormone concentrations are actually a modifiable risk factor for adverse neurodevelopmental outcomes of the offspring. In the only randomized controlled trial to date, the Controlled Antenatal Thyroid Study (CATS), women were randomized screening and subsequent treatment with 150 µg levothyroxine if the TSH concentration was above the 97.5th

percentile and/or the FT4 concentration was below the <2.5th percentile, or no screening or treatment at all.[67] The median start of treatment was in the 14th week of pregnancy and children underwent IQ testing at age 3 (390 and 404 children in the treated or untreated group, respectively).[67] The treatment did not lead to a higher mean IQ, or a lower proportion of children with an IQ below 85 (also not for the low FT4 or high TSH groups separately).[67] It has been postulated that these negative findings were due to the fact that treatment started too late (median 13 weeks), that IQ cannot be assessed reliably at age 3 or that the loss to follow-up was too high (24%). However, given that recent findings have indicated that also high maternal thyroid hormone concentrations are associated with lower offspring IQ, it is also possible that overtreatment may underlie the negative findings.[11,68] Currently, important follow-up studies of the CATS trial are being performed to identify any potential harms in women with high thyroid hormone concentrations due to treatment with levothyroxine.[69]

In addition, the results of an ongoing trial from the US, in which close to 100.000 women were screened and women with hypothyroxinemia or subclinical hypothyroidism were randomized to placebo or treatment with levothyroxine in a lower dosage than the CATS trial (50 or 75 µg) are currently awaited (NCT00388297). Unfortunately, this trial performed a power calculation to find a statistically significant difference of 5 IQ points. As cohort studies since the start of this trial have shown that low maternal thyroid function is associated with a 3 to 4 IQ point difference, it may be likely that this trial will be underpowered to show an expected, and relevant, difference in IQ.

Conclusion

The fact that the majority of studies that demonstrated associations of maternal thyroid function with neurocognitive outcomes come from only a few groups is seen as a limitation of the field, and the likeliness of a true association. In addition, it is likely that studied endpoints such as low IQ, brain morphology, autism and ADHD to a certain extent overlap. However, these outcomes are used to study the hypothesis that an optimal maternal thyroid function is necessary to provide adequate thyroxine to the fetus, which subsequently regulates fetal brain development. From this perspective, these different studied outcomes should perhaps all be considered as a proxy measure for the same process, that is intrauterine fetal brain development. Although it will be important to further study the association of maternal thyroid function with brain development across different populations, studying various neurodevelopment outcomes could be seen as a form of internal replication and may reveal more insights into the pathophysiology of thyroid hormone dependent processes of neurogenesis.

Although there is a wide basis of experimental and clinical evidence that supports the hypothesis that an optimal maternal thyroid function is important for offspring neurodevelopment, further studies are required to elucidate potential underlying mechanisms. By knowing the mechanisms we can focus on, or improve preventative measures and also further identify which subgroups of women with too low or too high thyroid hormones are high-risk individuals. In order to investigate this, it may be relevant to focus on factors that are both a determinant of thyroid function as well as offspring neurodevelopment such as iodine status and endocrine disrupting chemicals.[49,70-75] Furthermore, randomized controlled trials are necessary to investigate the benefits and potential harms of levothyroxine treatment during pregnancy and to define optimal treatment goals.

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SUMMARY

SAMENVATTING

SUMMARY

In **Chapter 1**, the pregnancy-specific changes that occur in thyroid physiology during pregnancy are described. The majority of these changes are further investigated in other chapters of this thesis, mainly focusing on hCG, TPO-antibodies, total T4 and other determinants. Subsequently, in this chapter the interpretation of thyroid hormone action as a continuous trait is described and we discuss methodological and physiological insights according to other literature. This chapter further focuses on the calculation of reference ranges, in particular the importance of calculating population-based reference ranges, it provides an overview of relevant studies on reference ranges for thyroid function during pregnancy and determinants of reference ranges are further elaborated on. Finally, a brief overview of the literature on commonly studied endpoints is provided.

Chapter 2 describes ethnic differences in various thyroid function measurement during pregnancy. It displays that, even within a population of pregnant women within the Rotterdam area, large ethnic differences in thyroid function exist and that these differences may impact reference ranges calculations. Subsequently, large differences in the proportion of thyroid disease diagnosis are shown between current reference ranges and ethnicity-specific reference ranges. Although this does not mean that use of ethnicity-specific reference ranges better identified women with thyroid disease, these analyses do exhibit the impact that ethnic variations may have in the comparison of thyroid studies worldwide.

The identification of a novel risk factor for maternal thyroid dysfunction is described in **Chapter 3**. Clinical observations and studies in mice highlight the importance of the thyroidal vasculature for thyroid function and this chapter shows that endogenous placental angiogenic factor sFlt1 and PlGF are associated with maternal thyroid function, disease and also the thyroidal response to hCG.

Chapter 4 aims to combine the predictive ability of various easily obtainable clinical characteristics that serve as a determinant of high TSH or low FT4 during pregnancy. Studies thus far have identified various of such characteristics as potential risk factors but never in a multivariate manner. Yet the fact that an individual patient with various characteristics is sitting in front of a physician requires studying the combination of risk factors. This chapter discusses that due to the strong association of TPO-antibodies with high TSH, the latter is hard to predict. For low FT4, this is different, and this chapter described the construction and validation of a prediction model for the biochemical presence of low FT4 exhibiting a relatively good accuracy and subsequently this is constructed into clinical prediction tools.

Chapter 5 describes the characteristics of hCG throughout pregnancy in a large population-based sample of pregnant women. Besides the classical role of hCG in maintaining the corpus luteum, hCG is an important thyroid stimulator. Thus, for clinical as well as research purposes it is important to define its baseline characteristics. The trajectory, reference ranges and various clinical determinants of hCG concentrations are studied and quantified, and specifically the associations of BMI, placental weight and smoking are described in detail.

Subsequently, in **Chapter 6**, the physiological aspects of thyroid stimulation by hCG during pregnancy are further studied. hCG is for the first time identified as an important risk factor for subclinical and overt hyperthyroidism as well as hypothyroxinemia, yet not for overt or subclinical hypothyroidism. In women with subclinical hypothyroidism, there is no association of hCG with FT4, suggesting a decreased thyroid functional capacity in these women. Hypothyroxinemia is considered as an enigmatic thyroid disease entity given its unexpected combination of TSH and FT4. This chapter also describes that in women with hypothyroxinemia the association of hCG with TSH is similar to that of euthyroid women. This suggests that hypothyroxinemia is a disease entity characterized by a high sensitivity to thyroid hormone, as opposed to the longstanding idea that it is mediated by (mild) iodine deficiency. These data give new insights into the pathogenesis of subclinical hypothyroidism and hypothyroxinemia.

For **Chapter 7**, the hypothesis that intrauterine adaption to thyroid hormone exposure may affect the set point of the hypothalamic-pituitary-thyroid axis is investigated by comparing maternal thyroid function during pregnancy with that of the offspring at birth and at 5 years of age. Furthermore, the parallel pathway of genetic overlap between the fetus and its mother is investigated using a risk score comprised of all known common genetic variants for thyroid function. It is described that there is a strong positive association between maternal TSH and child TSH at both time points, and that this is similar for FT4. Also, maternal FT4 is associated with cord blood TSH, indicating the placental transfer of maternal thyroxine to the fetus and its extent is quantified. Furthermore, the explained variability of this pathway in comparison to a genetic pathway is exhibited and practically no overlap was found suggesting two separate pathways of thyroid axis set point formation.

In line with the identification of angiogenic factors as a determinant for maternal thyroid function, **Chapter 8** discusses the association of angiogenic factors with thyroid function in the newborn, as measured in cord blood. This hypothesis focuses on the potential of angiogenesis differences to influence thyroid development. The results show that a more pro-angiogenic cord blood profile is associated with higher thyroid function and a lower risk of hypothyroxinemia. In addition, we identified that antiangiogenic sFlt1 is a strong determinant of transient hypothyroxinemia of prematurity, a very specific disease entity for which little determinants have been identified.

Chapter 9 describes a study in which we performed a systematic review of other studies that calculated reference ranges for thyroid function throughout childhood. This study reveals large differences in childhood references ranges, even when the same assays were used. We subsequently studied determinants of TSH and FT4 during childhood in the Generation R study and quantified to what extent these may influence reference ranges. We identified that sex, ethnicity and anthropometry were important determinants of TSH and FT4, which may also lead to a considerable difference in reference ranges.

In **Chapter 10**, we demonstrate that already high-normal maternal FT4 concentrations during pregnancy are associated with a higher risk of pre-eclampsia while overt gestational hyperthyroidism is associated with a higher risk of pregnancy-induced hypertension. No clinically relevant differences were identified in the trajectory of blood pressure during pregnancy.

Chapter 11 describes a study in which we show that high maternal TSH as well as TPO-antibody positivity is associated with a higher risk of premature delivery. Although this was a replication of previous studies, we also demonstrate that when TPOAb positive women are excluded from analyses, high TSH is no longer associated with a higher risk of premature delivery. Moreover, high TSH was not associated with the risk of premature rupture of membranes or spontaneous premature delivery suggesting that this may not be a direct effect on the biology underlying premature delivery. The results of this study also, for the first time, showed that hypothyroxinemia is associated with a higher risk of premature delivery. In contrast to high TSH, hypothyroxinemia was associated with premature rupture of membranes and the associations were amplified when spontaneous delivery was assessed.

Novel analyses showing that both low and high maternal FT4 concentrations during early pregnancy are associated with suboptimal neurodevelopment in the offspring is described in **Chapter 12**. Although previous studies have always focused on the effects of low thyroid hormone availability, this chapter demonstrates that there is an inverted U-shaped association of maternal FT4 concentrations with child IQ measured at a median age of 6 years. In addition, the association of maternal FT4 with gray matter and cortical thickness as assessed by MRI scans in a subset of children also showed an inverted U-shaped association. This chapter shows that maternal FT4 is associated with offspring IQ and brain morphology and indicates that overtreatment with levothyroxine during pregnancy may be possible.

A large Dutch individual participant based meta-analysis that investigates a functional cut-off limit for TPOAb positivity during pregnancy is described in **Chapter 13**. This study shows that from a population-based TPOAb cut-off of the 92nd percentile onwards, TSH concentrations and the risk of TSH >2.5mU/L start to increase. We also show that from this cut-off, the thyroidal stimulation by hCG starts to attenuate and that this is a cut-off that is specific for early pregnancy (when hCG concentrations are high). We show that a high percentage of women with TPO-antibody concentrations high enough to increase TSH are not considered TPO-antibody positive according to current cut-offs.

Chapter 14 describes that the variation in total T4 is larger than for FT4 and that total T4 explains less of the variation in TSH than FT4. Furthermore, this study describes that total T4 concentrations during early pregnancy are not associated with the risk of adverse outcomes at all or additive to FT4. Although some studies suggested that total T4 can be used as a proxy for thyroid function during pregnancy, the results of this study suggest that FT4 is the preferred measurement of thyroid function during pregnancy.

TPO-antibodies are the most important risk factor for thyroid dysfunction during pregnancy, in **Chapter 15**, it is reported that TPO-antibodies severely attenuate the thyroidal response to hCG. This was a cross-cohort consistency study of two prospective birth cohorts showing that higher hCG does not increase thyroid function in TPO-antibody positive women. TPO-antibodies have been shown to increase the risk of premature delivery, as described in chapter 11 of this thesis, and in the current chapter we show that premature delivery only occurs more frequently in TPO-antibody positive women that have a biochemical phenotype congruent with an abnormal thyroidal response to hCG.

Chapter 16 describes that hCG may be used to distinguish women that have a high thyroid function due to a non-gestational cause, i.e. autonomous thyroid hormone production due to toxic nodules, toxic multinodular goiter or TSH receptor stimulating antibodies, versus a gestational cause. This study shows that women with high thyroid function and high hCG do not have a higher risk of pre-eclampsia, in contrast however, women with high thyroid function despite low(-normal) hCG had a much higher risk of pre-eclampsia. This latter group likely also was the driving force behind the association in the whole population demonstrated in chapter 10 which indicates that only a subset of women with non-gestational causes underlying high(-normal) thyroid function have a higher risk of pre-eclamspia.

Using a similar approach, in **Chapter 17** the study that is described in Chapter 11 is revisited while also taking into account hCG when studying thyroid function. Since we show that the effects of TPO-antibodies on premature delivery were mediated via a decreased thyroid functional capacity in chapter 17, this chapter described a study that investigated the effects of hCG and thyroid function on premature delivery in TPOAb negative women. Although we were not able to identify consistent effects of high TSH on premature delivery in chapter 10, we now show that women with a high TSH and a high hCG have an up to 5-fold higher risk of premature delivery. This study suggests again that the addition of hCG adds to our clinical interpretation of thyroid function during pregnancy and allows for better clinical risk stratification.

In **Chapter 18 and 19**, the discussion section of this thesis, previous chapters are discussed, connected and put in context of other literature. In particular the most recent findings in the research field of thyroid and pregnancy, the methodological pitfalls for studies on offspring neurobehavioral outcomes and future perspective on the research field including endocrine disruptors and the need for international collaboration are emphasized.

SAMENVATTING

In **Hoofdstuk 1** worden de veranderingen die plaatsvinden in de schildklier fysiologie tijdens de zwangerschap beschreven. De meerderheid van deze processen worden in andere hoofstukken van dit proefschrift in meer detail uitgewerkt waarbij de nadruk gelegd wordt op hCG, TPO-antistoffen, totaal T4 en andere determinanten. Hier opeenvolgend wordt in dit hoofdstuk de interpretatie van schildklierhormoon actie als continue eigenschap beschreven en methodologische inzichten met betrekking tot klinisch-epidemiologische schildklier studies beschreven aan de hand van andere literatuur. Verder focust dit hoofdstuk zich op de methodologie achter de berekening van referentiewaarden, specifiek voor populatie-specifieke referentiewaarden, en geeft dit hoofdstuk een overzicht van relevante studies op dit gebied. Als laatste wordt een kort overzicht gegeven van de in het veld vaak bestudeerde eindpunten.

Hoofdstuk 2 beschrijft etnische verschillen in verschillende schildklierfunctie gerelateerde metingen tijdens de zwangerschap. Het hoofdstuk laat zien dat zelfs binnen een populatie van zwangere vrouwen in Rotterdam, grote etnische verschillen bestaan in onder andere TSH, totaal T4 en TPO-antistof positiviteit en, dat deze verschillen de referentiewaarden kunnen beïnvloeden. Dit hoofdstuk beschrijft vervolgens ook grote verschillen in de prevalentie van schildklierziekten als deze gediagnosticeerd worden op basis van de standaard referentiewaarde berekeningen in vergelijking met etniciteit specifieke referentiewaarden. Alhoewel deze resultaten niet direct suggereren dat het gebruik van etniciteit-specifieke referentiewaarden een verbetering van de klinische praktijk oplevert, impliceren deze wel dat de impact van etnische variatie in schildklierfunctie op de verschillen in schildklierstudies wereldwijd, groot kan zijn.

De identificatie van een nieuwe risicofactor voor maternale schildklier dysfunctie wordt beschreven in **Hoofdstuk 3**. Klinische observaties en dierstudies laten zien dat de vasculatuur van de schildklier een belangrijke rol speelt in het bewerkstelligen van optimale schildklierfunctie. In dit hoofdstuk laten wij zien dat endogene, placentaire angiogenese factoren (sFlt1 en PlGF) geassocieerd zijn met schildklierfunctie, schildklierziekte en de schildklier response op hCG stimulatie.

Hoofdstuk 4 beschrijft de constructie en replicatie van een klinisch predictiemodel voor hoog TSH en/of laag FT4 aan de hand van klinische patiënt eigenschappen. Verschillende studies hebben deze parameters reeds geïdentificeerd als potentiele risicofactoren, echter er is nog nooit onderzocht of deze factoren voorspellend zijn voor een lage schildklierfunctie. Echter, het is belangrijk om rekening te houden met het feit dat een arts een patiënt voor zich heeft die een combinatie van deze klinische risicofactoren kan presenteren. De resultaten van dit hoofdstuk tonen dat door de sterke associatie tussen TPO-antistoffen en hoog TSH, de laatstgenoemde moeilijk te voorspellen is. Risicofactoren voor een laag FT4 lieten een relatief goed vermogen zien heeft om laag FT4 te onderscheiden en dit model wordt vervolgens in een klinische risicoscore uiteen gezet.

Hoofdstuk 5 beschrijft de eigenschappen van hCG door de zwangerschap heen in een groot populatie cohort van zwangere vrouwen. Naast het feit dat hCG belangrijk is voor het behoud van het corpus luteum tijdens de vroege zwangerschap is, stimuleert hCG ook de schildklier vanwege de grote structurele gelijkenissen met TSH. Voor zowel klinische als onderzoeksdoeleinden is het daarom belangrijk om de basis eigenschappen van hCG concentraties in kaart te brengen. Het beloop van hCG tijdens de zwangerschap, referentiewaarden en ook klinische determinanten worden bestudeerd en gekwantificeerd, specifiek besproken wordt ook de relatie met BMI, placenta gewicht en roken.

Hier opeenvolgend, in **Hoofdstuk 6**, worden de fysiologische aspecten besproken met betrekking tot de schildklierstimulatie door hCG tijdens de zwangerschap. Er wordt voor het eerst beschreven dat hCG een belangrijke risicofactor is voor subklinische en overte hyperthyreoïdie alsmede

hypothyroxinemie, terwijl hCG niet geassocieerd is met overte en/of subklinische hypothyreoïdie. In vrouwen met een subklinische hypothyreoïdie is geen associatie aanwezig tussen hCG en FT4, wat een verlaagde functionele capaciteit van de schildklier suggereert. Ondanks dat hypothyroxinemie (laag FT4 met normaal TSH) als een onlogische, biochemische schildklierfunctie afwijking wordt beschouwd demonstreren we in dit hoofdstuk dat vrouwen met hypothyroxinemie een normale schildklier response op hCG stimulatie hebben. Dit suggereert dat, in tegenstelling tot de gedachte dat hypothyroxinemie veroorzaakt wordt door een (mild) jodiumtekort, hypothyroxinemie waarschijnlijk een uiting is van een lager schildklierhormoon *setpoint*.

In **Hoofdstuk 7** wordt de hypothese onderzocht dat de ontwikkeling van de hypothalamus-hypofyse-schildklier as mogelijk beïnvloed wordt door de blootstelling aan maternaal schildklierhormoon door de associatie tussen de maternale schildklierfunctie tijdens de zwangerschap en de schildklierfunctie van het kind tijdens de geboorte en op de leeftijd van gemiddeld 6 jaar te bestuderen. Parallel hieraan word ook de genetische overlap, onderzocht met behulp van een genetische risicoscore berekend aan de hand van alle reeds bekende veelvoorkomende genetische polymorfismen voor schildklierfunctie. In dit hoofdstuk laten we zien dat er een sterke associatie is tussen maternaal TSH en kind TSH, zowel tijdens de geboorte als op 6-jarige leeftijd, en dat hetzelfde geldt voor FT4. Hoger maternaal FT4 is tevens geassocieerd met lager navelstrengbloed TSH, wat aangeeft dat er placentair transport van maternaal geproduceerd thyroxine plaatsvindt. Verder wordt de verklaarde variantie van de adaptatie *pathway* met die van de genetische *pathway* vergeleken en laten we zien dat deze los van elkaar de schildklier as van het kind lijken te beïnvloeden.

Hoofdstuk 8 bediscussieerd de associatie van angiogenese factoren met schildklierfunctie in pasgeborenen, zoals gemeten in navelstrengbloed afgenomen vlak na de geboorte. In tegenstelling tot hoofdstuk 3, is de onderliggende hypothese voor deze studie dat angiogenese factoren middels de beïnvloeding van de schildklier vasculatuur effecten hebben op de vroege ontwikkeling van de schildklier. De resultaten laten zien dat een meer pro-angiogeen navelstrengbloed profiel geassocieerd is met een hogere schildklierfunctie en een lager risico op hypothyroxinemie. Daarbij wordt ook sFlt1 geïdentificeerd als sterke determinant voor de tijdelijke hypothyroxinemie geassocieerd met prematuriteit, een specifieke ziekte entiteit waarvoor nog weinig determinanten geïdentificeerd zijn.

In **Hoofdstuk 9** wordt een systematische review van studies die referentiewaarden tijdens de kinderleeftijd rapporteren beschreven. Deze studie laat grote verschillen in referentiewaarden voor TSH en FT4 zien, op basis van leeftijd, maar zelfs ook binnen gelijke leeftijdscategorieën en met het gebruik van gelijke assays. Om te kwantificeren hoeveel van deze variatie mogelijk verklaard kan worden door verschillen in schildklierfunctie determinanten tussen populaties hebben we vervolgens determinanten van TSH en FT4 geïdentificeerd in Generation R. De resultaten laten zien dat een groot gedeelte van de variatie binnen de populatie verklaard kan worden door verschillen karakteristieken van een populatie zoals geslacht, etniciteit, lengte en gewicht.

In **Hoofdstuk 10**, wordt beschreven dat hoog-normale FT4 waarden van de moeder tijdens de zwangerschap geassocieerd zijn met een hoger risico op pre-eclampsie en dat overte hyperthyreoïdie geassocieerd met een hoger risico op zwangerschapshypertensie. Voor het beloop van de bloeddruk tijdens de zwangerschap worden geen klinisch relevante verschillen beschreven.

Hoofstuk 11 beschrijft een studie waarin wij aantonen dat een hoog maternaal TSH en ook TPO-antistof positiviteit geassocieerd zijn met een hoger risico op vroeggeboorte. Dit resultaat is een replicatie van andere studies. Echter, in dit hoofdstuk beschrijven wij dat na exclusie van TPO-antistof positieve vrouwen, een hoog TSH niet langer geassocieerd is met vroeggeboorte. Tevens laten we zien dat hoog TSH niet geassocieerd is met vroeg gebroken vliezen of spontane vroeggeboorte wat suggereert dat hoog TSH zelf geen direct effect heeft op de onderliggende biologie van vroeggeboorte.

De resultaten van de studie laten ook voor het eerst zien dat hypothyroxinemie is geassocieerd met een hoger risico op vroeggeboorte. In contrast met de resultaten voor hoog TSH, is hypothyroxinemie wel geassocieerd met te vroeg gebroken vliezen en de associaties amplificeren als spontane vroeggeboorte wordt onderzocht.

Nieuwe resultaten die laten zien dat zowel hoge als lage FT4 concentraties tijdens de vroege zwangerschap geassocieerd zijn met een suboptimale neurocognitieve ontwikkeling van het kind wordt beschreven in **Hoofdstuk 12**. Ondanks dat de focus in dit onderzoeksveld praktisch altijd op de effecten van lage schildklierhormoon beschikbaarheid gericht was, beschrijft dit hoofdstuk dat er een omgekeerd U-vormig verband is van maternale FT4 concentraties met kind IQ gemeten op een mediane leeftijd van 6 jaar. Daarbij laten we precies dezelfde omgekeerd U-vormige associatie zien tussen maternaal FT4 en grijze stof massa en, volume van de cortex. De resultaten van deze studie laten voor het eerst zien dat maternaal FT4 geassocieerd is met de morfologie van het brein van het kind en suggereren dat overbehandeling met levothyroxine tijdens de zwangerschap mogelijk is.

Een *individual participant meta-analysis* waarin wij een functionele afkapwaarde voor TPOAb positiviteit tijdens de zwangerschap onderzocht hebben, wordt beschreven in **Hoofdstuk 13**. Deze studie laat zien dat bij een populatie afkapwaarde vanaf het 92^{e} percentiel, TSH concentraties en het risico op een TSH >2.5 mU/L verhogen. Deze studie laat tevens zien dat vanaf dezelfde afkapwaarde, de schildklierstimulatie door hCG af begint te nemen en dat deze afkapwaarde specifiek is voor de vroege zwangerschap (wanneer hCG concentraties hoog zijn). Verder laat de studie zien dat tot een kwart van alle vrouwen die TPO-antistof concentraties hebben die geassocieerd zijn met een lagere schildklierfunctie met gebruik van de huidige afkapwaarden niet als TPO-antistof positief worden beschouwd.

Hoofdstuk 14 beschrijft dat totaal T4 een grotere variatie heeft, en minder sterk geassocieerd is met TSH tijdens de vroege zwangerschap, in vergelijking met FT4. Verder laten we in dit hoofdstuk zien dat de totaal T4 concentratie niet, of niet beter dan FT4, geassocieerd is met het risico op ongewenste zwangerschapsuitkomsten. Dit druist in tegen eerdere studies die suggereerden dat totaal T4 gebruikt kan worden als proxy voor schildklierfunctie tijdens de zwangerschap.

TPO-antistoffen zijn de belangrijkste risicofactor voor schildklierdysfunctie tijdens de zwangerschap en in **Hoofdstuk 15** wordt beschreven dat in vrouwen die TPO-antistof positief zijn hogere hCG waarden niet geassocieerd zijn met een hogere schildklierfunctie. Omdat we dit vonden in zowel Generation R als de HAPPY studie, is dit een sterke aanwijzing dat schildklier auto-immuniteit leidt tot een sterk verminderde schildklier response tijdens stimulatie door hoge hCG concentraties. Zoals in hoofdstuk 11 beschreven wordt, hebben TPO-antistof positieve vrouwen een hogere risico op vroeggeboorte en in het huidige hoofdstuk wordt beschreven dat vroeggeboorte alleen plaatsvindt in TPO-antistof positieve vrouwen die een biochemisch fenotype hebben dat past bij een abnormale schildklier response op hCG (een lager dan verwacht FT4 voor de hCG concentratie).

Hoofdstuk 16 zet uiteen dat hCG gebruikt kan worden om vrouwen te onderscheiden die een hoog normale schildklierfunctie hebben dat niet door de zwangerschap zelf, maar waarschijnlijk door bijvoorbeeld autonome schildklierhormoon productie of door een toxische nodus, toxisch multinodulair struma of TSH receptor stimulerende antistoffen wordt veroorzaakt. Deze studie laat zien dat vrouwen met een hoge schildklierfunctie en hoge hCG concentraties geen hoger risico hebben op pre-eclampsie terwijl vrouwen met een hoge schildklierfunctie maar lage hCG concentraties een veel hoger risico op pre-eclampsie hebben. De laatste groep is zeer waarschijnlijk ook de groep geweest die de associaties zoals uiteengezet in hoofdstuk 10 veroorzaakten.

Met het gebruik van eenzelfde benadering wordt in **Hoofdstuk 17** de toegevoegde waarde van hCG in de associatie tussen schildklierfunctie en vroeggeboorte bestudeerd. Aangezien in hoofdstuk 17 al

beschreven wordt dat vrouwen met een verminderde schildklier capaciteit door TPO-antistof positiviteit een hoger risico op vroeggeboorte hebben, ligt de focus van deze studie op de TPO-antistof negatieve vrouwen. Ondanks dat in hoofdstuk 10 geen consistente associatie gevonden werd tussen TSH en vroeggeboorte, laat de studie beschreven in dit hoofdstuk zien dat vrouwen met een hoog-normaal TSH en een hoog hCG een tot wel 5 keer verhoogd risico hebben op vroeggeboorte. Deze studie suggereert wederom dat de toevoeging van hCG onze klinische interpretatie van schildklierfunctie tijdens de zwangerschap kan verbeteren en artsen de mogelijkheid geeft hun risicoschatting te verbeteren.

In **Hoofdstuk 18 en 19**, de discussie van dit proefschrift, worden de bovengenoemde hoofdstukken besproken, verbonden, en in de context van de huidige literatuur geplaatst. Voornamelijk de meest recente bevindingen in het onderzoeksveld dat zich richt op schildklier en zwangerschap, de methodologie van studies met betrekking tot schildklierfunctie en neurocognitieve gedragsuitkomsten en toekomst perspectieven voor het onderzoeksveld zoals endocriene verstoorders en het belang van internationale samenwerkingsverbanden worden besproken.

AUTHOR AFFILIATIONS

ERASMUS MC PHD PORTFOLIO

PUBLICATIONS NOT IN THIS THESIS

DANKWOORD

ABOUT THE AUTHOR

AUTHOR AFFILIATIONS

Department of Internal Medicine, section Endocrinology and Academic Center for Thyroid Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands.
Tim. I.M. Korevaar, M. Medici, L. Chaker, Mirjana Barjaktarovic, Ibrahim Onsesveren, W.Edward Visser, Theo J. Visser, Robin P. Peeters

Department of Obstetrics and Gynaecology, Erasmus University Medical Center, Rotterdam, the Netherlands.
Eric. A.P. Steegers, Willy Visser, Sarah Schalekamp-Timmermans

Department of Epidemiology, Erasmus University Medical Center, Rotterdam, the Netherlands.
Albert Hofman, Vincent W.V. Jaddoe, Henning Tiemeier, Romy Gaillard

Department of Pediatrics, Erasmus University Medical Center, Rotterdam, the Netherlands.
Sabine M. de Muinck Keizer-Schrama, Jacoba J. Bongers-Schokking, Vincent V.W. Jaddoe.

Department of Clinical Chemistry, Erasmus University Medical Center, Rotterdam, the Netherlands.
Yolanda B. de Rijke, Henk Russcher

Department of Child and Adolescent Psychiatry, Erasmus University Medical Center, Rotterdam, the Netherlands.
Henning Tiemeier, Ryan Muetzel, Tonya White

Department of Public Health, Erasmus University Medical Center, Rotterdam, the Netherlands.
Daan Nieboer, Ewout W. Steyerberg

Department of Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands.
Herbert Hooijkaas

Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.
H. Alec Ross

Department of Medical and Clinical Psychology, Tilburg University, the Netherlands.
Victor J. Pop

Laboratory of Clinical Chemistry and Haematology, Máxima Medical Centre, Veldhoven, the Netherlands.
Maarten A. Broeren

Department of Public Health, Academic Medical Center, Amsterdam, The Netherlands.
Tanja G. Vrijkotte

Department of Endocrinology, Academic Medical Center, Amsterdam, The Netherlands.
Peter H.L.T. Bisschop

Department of Reproductive Medicine, Academic Medical Center, Amsterdam, The Netherlands.
Mariëtte Goddijn

Institute of Metabolism and Systems Research, University of Birmingham, United Kingdom.
Kristien Boelaert

Department of Internal Medicine, Universitair Medisch Centrum Sint-Peters, Brussels, Belgium.
Kris Poppe

Department of Endocrinology and Metabolism, Academic Medical Centre, University of Amsterdam, the Netherlands.
Wilmar M. Wiersinga

ERASMUS MC PHD PORTFOLIO

PHD PORTFOLIO

Name of PhD student:	Drs. Tim I.M. Korevaar
Erasmus MC dept. of:	Internal Medicine, Endocrinology, Academic Center for Thyroid Diseases
PhD period:	September 2013 – February 2017
Promotors:	Prof.dr. Robin P. Peeters
	Prof.dr. Eric A.P. Steegers
Co-promotor:	Dr. Marco Medici

Training	Year(s)	ECTS
Courses		
Master of Science in Clinical Epidemiology, NIHES	2013-2015	70
MolMed Course and Workshop Basic and Translational Endocrinology	2014	2
Rotterdamse Internistendagen	2015,2016	0.7
Congress visits – oral presentation		
Dutch Endocrine Meeting, Noordwijk	2013	0.7
Science Days Internal Medicine, Antwerp	2013	0.7
European Thyroid Association, Leiden - *Young Investigator Session*	2013	0.7
British Endocrine Society, Liverpool, - *Young Investigator Session*	2014	0.7
Dutch Endocrine Meeting, Noordwijk	2014	0.7
Endocrine Society, San Diego – *Press released*	2015	0.7
Dutch Endocrine Meeting, Noordwijk	2015	0.7
Science Days Internal Medicine, Antwerp	2015	0.7
AAV Wetenschapsmiddag, Rotterdam	2015	0.7
International Thyroid Congress, Orlando	2015	0.7
Dutch Endocrine Meeting, Noordwijk	2016	0.7
Endocrine Society, Boston, USA – *Poster Preview Presentation*	2016	0.7
European Congress of Endocrinology, Munich – *Press released*	2016	0.7
European Thyroid Association, Copenhagen – *Young Investigator Session*	2016	0.7
American Thyroid Association, Denver	2016	0.7
British Endocrine Society, Brighton	2016	0.7
Congress visits – poster presentation		
Endocrine Society, San Francisco	2013	0.7
Science Days Internal Medicine, Antwerp	2014	0.7
European Thyroid Association, Santiago de Compostela	2014	0.7
European Society of Endocrinology, Dublin	2015	0.7
Endocrine Society, Boston – *Presidential Poster Competition*	2016	0.7

Congress visits – other

EUthyroid kick-off meeting, Vienna	2015	0.7
Dutch Thyroid Research Club, Amsterdam	2014-2016	0.7

Teaching

Invited lectures

CEDAM Seminar, University of Birmingham *Translating gestational thyroid function physiology into clinical research.*	2015	0.3
Rotterdam Science Festival *Gestational iodine deficiency of the mother and impaired offspring brain development.*	2015	0.1
ENDO Retreat, Rotterdam *Thyroid function during pregnancy and child development.*	2015	0.1
Dutch Thyroid Club, Groningen *hCG stimulation of thyroid function.*	2016	0.1
Iodine Global Network Symposium - Iodine and Pregnancy, London *EUthyroid – Maternal iodine and offspring neurodevelopment.*	2016	0.1
Clinical Update on Endocrine Disorders in Pregnancy, Stockholm *High gestational thyroid function: causes and consequences.*	2016	0.6
Controversia in tiroidologia neonatale e maternal, Pisa *Maternal thyroid function and child thyroid function, IQ and brain morphology.*	2016	0.6
European Society of Endocrinology Summer School, Bregenz *Maternal-placental determinants of offspring thyroid function.*	2016	1
Int. Congress of Endocrinology – Chinese Society of Endocrinology, Beijing Symposium: *Gestational thyroid function and offspring brain development: clinical evidence on cognition and behavior.* Meet the expert: *Thyroid and pregnancy.*	2016	1.2
International Congress of Endocrine Disorders, Tehran Symposium: *Screening of thyroid disorders in pregnant women.* Plenary: *Gestational thyroid function and offspring brain development.*	2016	1
Thyroid Grand Course, Sofia *Thyroid and pregnancy, a clinical update.*	2016	0.6
British Endocrine Society, Brighton *Interpretation of gestational thyroid function measurements: clinical risks and physiological considerations.*	2016	1

Medical curriculum and (co-)supervision of research projects

First year medical students, clinical courses on thyroid and Cushing's disease	2013-2015	1
Clinical technology students, clinical course on thyroid disease	2016	0.5
Mirjana Barjaktarovic, PhD student – *the combined role of hCG and thyroid function on pregnancy and child outcomes.*	2014-2017	3
Deborah Levie, PhD student - *maternal iodine status during pregnancy with thyroid function and fetal brain development (EUthyroid project).*	2015-2017	2
Ibrahim Önsesveren, MSc student - *determinants of childhood thyroid function.*	2016	1
Toyah Janssen, MSc student – *early childhood thyroid function and brain morphology.*	2016-2017	2
Elena Pavleska, MSc student - *bivariate Mendelian rand. of thyroid function and BMI.*	2015	1
Melissa van der Windt, medical student – *thyroid function with quality of life in treated thyroid cancer patients.*	2015	1
Karlien Veldscholte, medical student – *thyroid function and bone accrual in childhood.*	2016-2017	1

Other

Staff editor for the Erasmus Journal of Medicine	2016-2017	2

Academic activities

Associate Editor for Clinical Thyroidology	2016-2017	8
Program Committee Member, American Thyroid Association	2016-2019	0.2
Clinical Endocrinology Update Steering Committee Member; Endocrine Society	2016-2019	0.2
Editorial Board member of Thyroid Research	2016-2017	1
Member of the Scientific Advisory Board of the World Iodine Association	2015-2017	1
Co-founder and Executive Committee Coordinator of the Collaboration for Elucidating Clinical Thyroid Disruption by EDCs; *an international collaboration of cohort studies aiming to study the clinical effects of endocrine disrupting compounds on thyroid function.*	2016-2017	3
Co-founder and Executive Committee Coordinator of the Consortium on Thyroid and Pregnancy: *an international collaboration of cohort studies aiming to combine worldwide data and study the association of maternal thyroid function with adverse outcomes.*	2016-2017	5
Peer reviews (55) for *the BMJ, Lancet Diabetes & Endocrinology, Annals of Internal Medicine, Thyroid, Clinical Endocrinology, Nature Scientific Reports, European Thyroid Journal, Journal of the Endocrine Society, BMC Endocrine Disorders, Molecular and Cellular Endocrinology, International Journal of Public Health, Endocrinology Diabetes and Metabolism Case Reports, Wellbeing for Women Charity, World Cancer, Research Fund International, Oxford University Press, PLoS One (verification at: http://publons.com/author/1004492).*	2013-2017	10

Grants and prizes

Research grants

The Dutch National Thyroid Association, *co-applicant*: "Thyroid and pregnancy; consequences for the mother" (€ 2,500).	2013
ERAWEB PhD grant, *co-applicant*: Grant for full PhD student in our group (MB).	2014
Horizon2020-PHC-06-2014, *co-applicant*: "EUthyroid – Towards the elimination of iodine deficiency and preventable thyroid-related diseases in Europe" (€ 2,999,950 for total proposal, €183,340 for our group).	2015
KNAW Ter Meulen Beurs, *personal grant* "Disruption of the Fetal-Maternal Thyroid Hormone Axis by Endocrine Disrupting Chemicals and the Risk of Adverse Child Outcomes" (€10,000).	2016
Stichting De Drie Lichten Beurs 2016 *personal grant* "Wat is de (synergistische) invloed van maternale endocriene verstoorders en schildklierfunctie tijdens de zwangerschap op hersenontwikkeling van het kind?" (€5,600)	2017
Sophia Foundation SSWO grant, *head applicant*, "Unraveling pathophysiological mechanisms of thyroid hormone dependent brain development" (€ 69,000).	2017

Award and prizes

British Thyroid Association Award	2014
Best Oral Communication Award, SfE BES	2014
Outstanding Abstract Award, Endocrine Society	2015
Poster Prize, European Congress of Endocrinology	2015
ESE Young Investigator Award	2016
Society for Endocrinology Clinical Lectureship Early Career Award	2016

Meeting and travel grants

ENDO Early Career Forum travel grant	2013
Society for Endocrinology Conference grant	2013
Trustfonds Conference grant	2013
European Thyroid Association Young Investigator Travel Award	2013
Society for Endocrinology Conference and travel grant BES	2014
Trustfonds Conference grant	2014
Society for Endocrinology Conference grant	2015
Trustfonds Conference grant	2015
International Thyroid Conference Trainee Grant Program	2015
European Thyroid Association Travel Grant	2015
Goodlife Healthcare Travel Award of the Dutch Endocrine Society	2015
Society for Endocrinology Conference Grant	2016
European Thyroid Association Young Investigator Travel Award	2016
American Thyroid Association Trainee Grant Program	2016

Data collection tasks

Generation R (general tasks)	2013-2017	60
Labwork hCG measurements (~8500 samples)	2013-2014	6
Labwork EUthyroid iodine measurements (~1500 samples)	2016	3

PUBLICATIONS NOT IN THIS THESIS

Peer reviewed publications

1. TI Korevaar, RP Peeters. Letter to the Editor: Methodological comments on the study by Negro et al. entitled "Impact of Levothyroxine in Miscarriage and Preterm Delivery Rates in First Trimester Thyroid Antibody-Positive Women with TSH<2.5mIU/L". *J Clin Endocrinol Metab* 2016;101:L101-L2.

2. L Chaker, FJ Wolters, D Bos, TI Korevaar, A Hofman, A van der Lugt, PJ Koudstaal, OH Franco, A Dehghan, MW Vernooij, RP Peeters, MA Ikram. Thyroid function and the risk of dementia: The Rotterdam Study. *Neurology* 2016;87:1688-95.

3. FV van Zijl, DA Monserez, TI Korevaar, O Bugter, MH Wieringa, RJ Baatenburg de Jong, JA Hardillo. Postoperative value of serum squamous cell carcinoma antigen as a predictor of recurrence in sinonasal inverted papilloma. *Clin Otolaryngol* 2016.

4. M Barjaktarovic, TI Korevaar, VW Jaddoe, YB de Rijke, TJ Visser, RP Peeters, EA Steegers. Human chorionic gonadotropin (hCG) concentrations during the late first trimester are associated with fetal growth in a fetal sex-specific manner. *Eur J Epidemiol* 2016.

5. CP Teuwen, TI Korevaar, RL Coolen, T van der Wel, CA Houck, R Evertz, A Yaksh, JW Roos-Hesselink, AJ Bogers, NM de Groot. Frequent atrial extrasystolic beats predict atrial fibrillation in patients with congenital heart defects. *Europace* 2016.

6. L Chaker, S Ligthart, TI Korevaar, A Hofman, OH Franco, RP Peeters, A Dehghan. Thyroid function and risk of type 2 diabetes: a population-based prospective cohort study. *BMC Med* 2016;14:150.

7. TI Korevaar, PN Taylor, CM Dayan, RP Peeters. An Invitation to Join the Consortium on Thyroid and Pregnancy. *Obstet Gynecol* 2016;128:913.

8. L Chaker, TI Korevaar, M Medici, AG Uitterlinden, A Hofman, A Dehghan, OH Franco, RP Peeters. Thyroid Function Characteristics and Determinants: The Rotterdam Study. *Thyroid* 2016;26:1195-204.

9. H Tiemeier, TI Korevaar. A New Modifiable Risk Factor for Schizophrenia? *Biol Psychiatry* 2016;79:950-1.

10. ET Massolt, M van der Windt, TI Korevaar, BL Kam, JW Burger, GJ Franssen, I Lehmphul, J Kohrle, WE Visser, RP Peeters. Thyroid hormone and its metabolites in relation to quality of life in patients treated for differentiated thyroid cancer. *Clin Endocrinol (Oxf)* 2016;85:781-8.

11. ET Massolt, G Effraimidis, TI Korevaar, WM Wiersinga, WE Visser, RP Peeters, HA Drexhage. Aberrant Levels of Hematopoietic/Neuronal Growth and Differentiation Factors in Euthyroid Women at Risk for Autoimmune Thyroid Disease. *PLoS One* 2016;11:e0153892.

12. AM Leung, TI Korevaar, RP Peeters, RT Zoeller, J Kohrle, LH Duntas, GA Brent, BA Demeneix. Exposure to Thyroid-Disrupting Chemicals: A Transatlantic Call for Action. *Thyroid* 2016;26:479-80.

13. C Zevenbergen, TI Korevaar, A Schuette, RP Peeters, M Medici, TJ Visser, L Schomburg, WE Visser. Association of antiepileptic drug usage, trace elements and thyroid hormone status. *Eur J Endocrinol* 2016;174**:**425-32.

14. M Medici, E Porcu, G Pistis, A Teumer, SJ Brown, RA Jensen, R Rawal, GL Roef, TS Plantinga, SH Vermeulen, J Lahti, MJ Simmonds, LL Husemoen, RM Freathy, BM Shields, D Pietzner, R Nagy, L Broer, L Chaker, TI Korevaar, MG Plia, C Sala, U Volker, JB Richards, FC Sweep, C Gieger, et al. Identification of novel genetic Loci associated with thyroid peroxidase antibodies and clinical thyroid disease. *PLoS Genet* 2014;10**:**e1004123.

15. M Medici, N Direk, WE Visser, TI Korevaar, A Hofman, TJ Visser, H Tiemeier, RP Peeters. Thyroid function within the normal range and the risk of depression: a population-based cohort study. *J Clin Endocrinol Metab* 2014;99**:**1213-9.

16. GA Godoy, TI Korevaar, RP Peeters, A Hofman, YB de Rijke, JJ Bongers-Schokking, H Tiemeier, VW Jaddoe, R Gaillard. Maternal thyroid hormones during pregnancy, childhood adiposity and cardiovascular risk factors: the Generation R Study. *Clin Endocrinol (Oxf)* 2014;81**:**117-25.

17. EA Pereira, P Plaha, A Chari, M Paranathala, N Haslam, A Rogers, T Korevaar, D Tran, R Olarinde, N Karavitaki, AB Grossman, SA Cudlip. Transsphenoidal pituitary surgery in the elderly is safe and effective. *Br J Neurosurg* 2014;28**:**616-21.

18. TI Korevaar, F Ragazzoni, A Weaver, N Karavitaki, AB Grossman. IGF2-induced hypoglycemia unresponsive to everolimus. *QJM* 2014;107**:**297-300.

19. TI Korevaar, AB Grossman. Pheochromocytomas and paragangliomas: assessment of malignant potential. *Endocrine* 2011;40**:**354-65.

20. T Korevaar, JA Wass, AB Grossman, N Karavitaki. Disconnection hyperprolactinaemia in nonadenomatous sellar/parasellar lesions practically never exceeds 2000 mU/l. *Clin Endocrinol (Oxf)* 2012;76**:**602-3.

Other publications

22. TIM Korevaar, G Ntali, N Karavitaki. Craniopharyngiomas: An overview. *Springerlink,Tumours of the central nervous system,* Volume 9, 2012, pp 241-247.

23. TIM Korevaar, M Medici, YB de Rijke, H Russcher, RP Peeters. Reply to: Circulating vascular growth factors may influence fetal thyroid function. *Clin Thyroidol 2015;27:126–128.*

Clinical Thyroidology Commentaries (2016):

Jan. Perchlorate Is Negatively Associated with Maternal Thyroid Function during Pregnancy.

Feb. The Use of Genetics May Infer Causality in the Association of Body Composition with Thyroid Function.

March Translating differences in clinical fertility state to intracellular gene expression of thyroid hormone receptors, function and fertility markers.

April Predicting recurrence after block and replace therapy for Graves' hyperthyroidism – the GREAT way.

May In healthy women, variation of TSH in the normal range or thyroid autoimmunity is not associated fecundity, pregnancy loss or live birth.

June Normalization of TSH in women with mild subclinical hypothyroidism during pregnancy bears a low risk of overtreatment.

July Using genetics to prevent antithyroid drug induced agranulocytosis.

Aug. Thyroid autoimmunity in euthyroid women: no effects on early reproduction but confirmed consistency for risk of miscarriage and preterm delivery.

Sept. High TSH and TPOAb positivity are associated with a higher risk of gestational diabetes mellitus in a synergistic manner.

Oct. Prevalence of Differentiated Thyroid Cancer Found in Autopsy Studies Has Not Increased since 1970

Nov. Fine Particle Air Pollution May Alter Newborn Thyroid Function

Dec. Thyroid Function Changes Already Within the Normal Range Are a Risk Factor for Type 2 Diabetes Mellitus.

DANKWOORD

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ABOUT THE AUTHOR

Tim Korevaar was born on May 9th 1988 in Leidschendam, the Netherlands. He completed high school at the Scholengemeenschap Dalton Voorburg in 2006 and started medical school at the Erasmus University Medical Center in that same year. After a second-year elective in endocrinology under supervision of Professor Aart-Jan van der Lelij, he could not get rid of the urge to become an endocrinologist. During medical school, he spent a 7-month research internship at the Oxford Center for Diabetes, Endocrinology and Metabolism studying disease of the pituitary and adrenals mentored by Dr. Niki Karavitaki and Professor Ashley Grossman. Even further committed to endocrinology, he started a research project with Professor Robin Peeters and Professor Theo Visser during his clerkships. This project formed the beginning of his PhD trajectory that focused on studying the effects of thyroid function during pregnancy using a translational, clinical epidemiological approach. Finishing medical school in the fall of 2013 allowed him to perform fulltime research. In the first years of his PhD, Tim obtained a Master's degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences. During the remainder of his PhD, he had the privilege to receive six international awards, visit various conferences as an abstract presenter or invited speaker and become part of the H2020 EUthyroid project. In the final year of his PhD, he initiated and co-founded an international research collaboration (the Consortium on Thyroid and Pregnancy) and was awarded with the KNAW ter Meulen Beurs, Stichting de Drie Lichten Beurs and Sophia Stichting (SSWO) beurs. These will enable him to spend a semester as a visiting postdoctoral scientist at the Harvard School of Public Health with Professor Russ Hauser, and the Brigham and Women's Hospital with Professor Erik Alexander (TMB and DLB) and, extend some of the current research efforts with his collaborators (SSWO). Upon his return in the fall of 2017, he aims to start his clinical training in internal medicine and endocrinology and, extend the work of his PhD to fill clinical knowledge-gaps on the role of thyroid function in reproduction.